

APPENDIX 4 (response #4)

(Mouse lymphoma and in-vivo micronucleus assay data)

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2005-4101B1-01-02-APPENDIX-4-MENTOR

FINAL REPORT

Study Title

**MICRONUCLEUS CYTOGENETIC ASSAY IN MICE CONDUCTED
WITH TEST ARTICLE EXTRACTS**

Test Article

300cc Siltex Moderate Profile Gel-filled Mammary Prosthesis
Catalog # 354-3007, Lot # 257949

Authors

Ramadevi Gudi, Ph.D.
Ljubica Krsmanovic, Ph.D.

Study Completion Date

19 May 2003

Performing Laboratory

[REDACTED]

Laboratory Study Number

AA73XP.123201.BTL

Sponsor

Mentor Corporation
201 Mentor Drive
Santa Barbara, CA 93111

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[REDACTED]

STATEMENT OF COMPLIANCE

Study AA73XP.123201.BTL was conducted in compliance with the U.S. FDA Good Laboratory Practice Regulations as published in 21 CFR 58, the U.S. EPA GLP Standards 40 CFR 160 and 40 CFR 792, the UK GLP Compliance Programme, the Japanese GLP Standard and the OECD Principles of Good Laboratory Practice in all material aspects with the following exceptions:

The identity, strength, purity and composition or other characteristics to define the test article have not been determined by the testing facility or the Sponsor.

The stability of the test article has not been determined by the testing facility or the Sponsor.

Analyses to determine the uniformity or concentration of the test article extracts and their stability have not been determined by the testing facility or the Sponsor.

Ramadevi Gudi

Ramadevi Gudi, Ph.D.
Study Director

19 May 2003

Date

Richard J

BioReliance Study Management

19 May 2003

Date

Quality Assurance Statement

Study Title: MICRONUCLEUS CYTOGENETIC ASSAY IN MICE CONDUCTED WITH TEST ARTICLE EXTRACTS

Study Number: AA73XP.123201.BTL

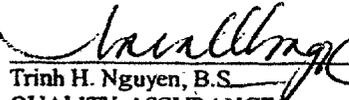
Study Director: Ramadevi Gudi, Ph.D.

This study has been divided into a series of in-process phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment records, etc., are examined in order to assure that the study is performed in accordance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR 58), the U.S. EPA GLPs (40 CFR 792 and 40 CFR 160), the UK GLP Regulations, the Japanese GLP Standard, and the OECD Principles of Good Laboratory Practice and to assure that the study is conducted according to the protocol and relevant Standard Operating Procedures.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

| | |
|---------------------|---|
| Inspect On Phase | 03-Apr-03 - 03-Apr-03 To Study Dir 03-Apr-03 To Mgmt 04-Apr-03 Protocol Review |
| Inspect On Phase | 15-Apr-03 - 15-Apr-03 To Study Dir 15-Apr-03 To Mgmt 17-Apr-03 Test and/or control material administration |
| Inspect On Phase | 07-May-03 - 08-May-03 To Study Dir 08-May-03 To Mgmt 09-May-03 Draft Report |
| Inspect On Phase | 16-May-03 - 16-May-03 To Study Dir 16-May-03 To Mgmt 19-May-03 Draft to Final Report |

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.


Trinh H. Nguyen, B.S.
QUALITY ASSURANCE

19 May 2003
DATE

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**MICRONUCLEUS CYTOGENETIC ASSAY IN MICE CONDUCTED
WITH TEST ARTICLE EXTRACTS**

STUDY INFORMATION

Sponsor: **Mentor Corporation
201 Mentor Drive
Santa Barbara, CA 93111**

Authorized Representative: **Philip Yang**

Testing Facility: 

BioReliance Study No.: **AA73XP.123201.BTL**

Test Article I.D.: **300cc Siltex Moderate Profile Gel-filled Mammary
Prosthesis (Catalog # 354-3007, Lot # 257949)**

Test Article Description by
BioReliance: **White opaque gel filled sac with a harder outside shell**

Storage Conditions: **Room temperature; protected from exposure to light and
moisture**

Saline Test Article Extract
Description: **Clear, colorless liquid**

Corn oil Test Article Extract
Description: **Clear, yellow liquid**

Test Article Receipt/Login: **28 March 2003/31 March 2003**

Study Initiation: **03 April 2003**

Experimental Start Date: **15 April 2003**

Experimental Completion Date: **04 May 2003**

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SUMMARY

The test article, 300cc Siltex Moderate Profile Gel-filled Mammary Prosthesis, was extracted in both saline and corn oil and each extract was tested in the mouse micronucleus assay. The assay was designed to evaluate the potential of the test article extracts to increase the incidence of micronucleated polychromatic erythrocytes in bone marrow of male and female ICR mice.

In this study, male and female ICR mice (5/group/sacrifice time) were exposed to undiluted saline or corn oil test article extracts, saline or corn oil extraction blanks (negative controls) or to the positive control article (cyclophosphamide monohydrate, CP). All dosing formulations were administered at a constant volume of 20 mL/kg by a single intraperitoneal injection. Animals were observed for clinical signs of toxicity after dose administration and daily thereafter until the scheduled sacrifice time. Bone marrow cells (polychromatic erythrocytes, PCEs and normochromatic erythrocytes, NCEs), collected 24 and 48 hours after treatment, were examined microscopically for the presence of micronuclei (MPCEs and MNCEs). The incidence of MPCEs per 10000 PCEs (2000 PCEs/animal) was determined for all treatment groups. Results for CP, saline and corn oil test article extracts were compared to the saline and corn oil extraction blanks to determine significance at the $p \leq 0.05$ level using Kastenbaum-Bowman Tables.

No mortality or clinical signs were observed during the course of the study. All mice appeared normal following dose administration and thereafter until the scheduled sacrifice time.

No appreciable reductions in the ratio of polychromatic erythrocytes to total erythrocytes were observed in the test article extract-treated groups relative to the respective negative controls suggesting that test article extracts did not inhibit erythropoiesis. Additionally, no significant increase in micronucleated polychromatic erythrocytes in the test article saline or corn oil extract-treated groups relative to the respective negative controls was observed in male or female mice at 24 or 48 hours after dose administration ($p > 0.05$, Kastenbaum-Bowman Tables).

The results of the assay indicate that under the conditions described in this report, the saline and corn oil extracts of the test article did not induce a significant increase in micronucleated polychromatic erythrocytes in either male or female ICR mice. Therefore, saline and corn oil extracts of 300cc Siltex Moderate Profile Gel-filled Mammary Prosthesis were concluded to be negative in the micronucleus test.

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PURPOSE

The purpose of this study was to evaluate the clastogenic potential of the saline and corn oil extracts of the test article as measured by their ability to induce micronucleated polychromatic erythrocytes in mouse bone marrow.

The study protocol is included in Appendix III.

CHARACTERIZATION OF TEST AND CONTROL ARTICLES

Test Article Description

The test article, 300cc Siltex Moderate Profile Gel-filled Mammary Prosthesis was received by BioReliance on 28 March 2003 and was assigned the code number AA73XP. The test article was characterized by the Sponsor as a sterile medical device made of silicone gel and silicone elastomers, that should be stored at room temperature. The test article catalog number of 354-3007, lot number of 257949 and an expiration date of 31 March 2008 were provided by the Sponsor. Upon receipt, the test article was described as white opaque gel and was stored at room temperature, protected from exposure to light and moisture. Prior to extraction procedure, the test article was described as a white opaque, gel filled sac with a harder outside shell.

Test Article Extraction Procedure

The test article was extracted in each extraction medium, saline and corn oil. The test article was extracted based on a ratio of 0.2 g per 1 mL of extraction medium. The test article was cut into small pieces, through all layers, placed in an extraction vessel and immersed in the required volume of extraction medium. The headspace of the each extraction vessel was purged with nitrogen gas and capped tightly. Each test article extract was incubated for 72 ± 2 hours at $50 \pm 2^\circ\text{C}$ with shaking. At the end of the extraction period, the vessels were cooled to room temperature and shaken vigorously for several minutes. Then, the extracts were aseptically decanted into sterile, glass vessels and stored at room temperature until use. Saline and corn oil extraction blanks were prepared in the same manner, without the addition of test article. All extracts and extraction blanks were used within 24 hours of preparation.

Characterization of Extraction Media and Positive Control

Cyclophosphamide, (CP, CAS No. 6055-19-2), was obtained from Sigma Chemical Company and was dissolved in sterile distilled water at a concentration of 2.5 mg/mL for use as the positive control. Saline was obtained from Baxter Healthcare, 0.9%, USP grade (CAS No. 7647-14-5) and corn oil was obtained from Sigma Chemical Company (CAS No. 8001-30-1). Test article extraction media (saline and corn oil) and positive control articles have been characterized as per the Certificates of Analysis on file with the testing facility. The stability of

the control articles and their mixtures was demonstrated by acceptable results that met the criteria for a valid test.

MATERIALS AND METHODS

Test System

ICR mice were obtained from Harlan Sprague Dawley, Inc., Frederick, MD and were received on 08 April 2003. At the initiation of the study, the mice were approximately 6 to 8 weeks old. Animal body weights recorded at randomization were within the following ranges:

Micronucleus Assay: Male: 28.4 – 33.6 g
 Female: 25.7 – 30.1 g

Individual animal body weights at dose administration, and mean body weight and standard deviation for each group are presented in Appendix II.

Animal Receipt and Quarantine

Mice were obtained from a source monitored for evidence of ectoparasites, endoparasites, pathogenic bacteria, mycoplasmas, and appropriate murine viruses and were quarantined for no less than 5 days after receipt. The mice were observed each working day for signs of illness, unusual food and water consumption, and other conditions of poor health. The animals were judged to be healthy prior to utilization in the assay.

Animal Care

The mice were housed in an AAALAC-accredited facility with a controlled environment of $72\pm 3^{\circ}\text{F}$ temperature, $50\pm 20\%$ relative humidity, and a 12 hour light/dark cycle. Mice of the same sex were housed up to five per cage in polycarbonate cages which were maintained on stainless steel racks equipped with automatic watering manifolds and which were covered with filter material. Heat-treated hardwood chips were used for bedding. Mice had free access to certified laboratory rodent chow (Harlan 2018C Certified Global Rodent Diet) which had been analyzed for environmental contaminants and to tap water (Washington Suburban Sanitary Commission, Potomac Plant). There were no contaminants in the feed which were considered to have influenced the results of the study. The water used in the study met USEPA drinking water standards and is monitored at least annually for levels of organophosphorus pesticides, metals, coliform bacteria and other contaminants.

Micronucleus Assay

The micronucleus assay was conducted using established and validated procedures (Heddle, 1973; Hayashi et al., 1994; Mavourmin et al., 1990). The mice were assigned to nine experimental groups of five males and five females each according to a computer-generated

program, which is based on distribution according to body weight. Each mouse was given a sequential number and identified by an ear tag. The study design was as follows:

| Treatment (20 mL/kg) | Number of Mice Per Sex Dosed | Number of Mice Per Sex Used for Bone Marrow Collection After Dose Administration | |
|---------------------------------|------------------------------|--|-------|
| | | 24 hr | 48 hr |
| Saline Extraction Blank | 10 | 5 | 5 |
| Corn oil Extraction Blank | 10 | 5 | 5 |
| Saline Test Article Extract | 10 | 5 | 5 |
| Corn oil Test Article Extract | 10 | 5 | 5 |
| Positive Control: CP (50 mg/kg) | 5 | 5 | 0 |

Dose Administration

Undiluted test article extracts, the extraction blanks, or the positive control were administered by a single intraperitoneal injection at a constant volume of 20 mL/kg body weight. Intraperitoneal injection was selected to maximize delivery of the test article to the test system. All mice in the experimental and control groups were weighed immediately prior to dose administration, and the dose volume was based on individual body weights. Mice were observed after dose administration for clinical signs of chemical effect.

Bone Marrow Collection and Slide Preparation

Twenty-four and 48 hours after dose administration, five animals per test article extract-treated and negative control groups were sacrificed by carbon dioxide asphyxiation. Immediately following sacrifice, the femurs were exposed, cut just above the knee, and the bone marrow was aspirated into a syringe containing fetal bovine serum. The bone marrow cells were transferred to a capped centrifuge tube containing approximately 1 mL fetal bovine serum. The bone marrow cells were pelleted by centrifugation at approximately 100 x g for five minutes and the supernatant was drawn off, leaving a small amount of serum with the remaining cell pellet. The cells were resuspended by aspiration with a capillary pipet and a small drop of bone marrow suspension was spread onto a clean glass slide. Two slides were prepared from each mouse. Each slide was identified by experiment and animal numbers. The slides were fixed in methanol, stained with May-Gruenwald-Giemsa and permanently mounted.

Scoring for Micronuclei

Bone marrow cells, polychromatic erythrocytes (PCEs) and normochromatic erythrocytes (NCEs) were analyzed for the presence of micronuclei. Polychromatic erythrocytes are young, immature red blood cells that stain bluish while normochromatic erythrocytes or normocytes are mature red blood cells that stain pink. Micronuclei are round, darkly-staining nuclear fragments with a sharp contour and diameters usually from 1/20 to 1/5 of an erythrocyte. Micronuclei can occur in both PCEs (MPCEs) and NCEs (MNCEs).

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Slides were coded using a random number table by an individual not involved with the scoring process. Using medium magnification, an area of acceptable quality was selected such that the cells were well spread and stained. Using oil immersion, 2000 polychromatic erythrocytes per animal were scored for the presence of micronuclei. The number of micronucleated normochromatic erythrocytes in the field of 2000 polychromatic erythrocytes was enumerated. The proportion of polychromatic erythrocytes to total erythrocytes was also recorded per 1000 erythrocytes.

Evaluation of Test Results

The incidence of micronucleated polychromatic erythrocytes per 2000 polychromatic erythrocytes was determined for each mouse and treatment group. Statistical significance was determined using the Kastenbaum-Bowman tables which are based on the binomial distribution (Kastenbaum and Bowman, 1970). All analyses were performed separately for each sex and sampling time.

In order to quantify the proliferation state of the bone marrow as an indicator of bone marrow toxicity, the proportion of polychromatic erythrocytes to total erythrocytes was determined for each animal and treatment group.

All conclusions were based on sound scientific judgement; however, as a guide to interpretation of the data, the test article extract was considered to induce a positive response if a statistically significant increase in micronucleated polychromatic erythrocytes was observed relative to the negative controls ($p \leq 0.05$, Kastenbaum-Bowman Tables) at any sampling time. The test article extract was considered negative if no statistically significant increase in micronucleated polychromatic erythrocytes above the concurrent extraction blank control was observed at all sampling times.

Criteria for a Valid Test

The mean incidence of micronucleated polychromatic erythrocytes must not exceed 5/1000 polychromatic erythrocytes (0.5%) in the negative (extraction blank) control. The incidence of micronucleated polychromatic erythrocytes in the positive control group must be significantly increased relative to the negative control group ($p \leq 0.05$, Kastenbaum-Bowman Tables).

Records and Archives

All raw data, protocol, and a copy of all reports will be maintained according to Standard Operating Procedure OPQP3040 by the BioReliance RAQA unit headquartered at: BioReliance, 14920 Broschart Rd., Rockville, MD 20850. Per this SOP, paper records will be retained for at least three years after which time the Sponsor will be contacted for a decision as to the final disposition of the materials. All study materials returned to the Sponsor or destroyed will first be copied and the copy will be retained in the BioReliance archives for a minimum of 10 years.

Deviations

No known deviations from the protocol or assay method SOPs occurred during the conduct of this study.

RESULTS AND DISCUSSION

Justification for Choice of Extraction Media

Saline and corn oil were selected as the extraction media because of their compatibility with the test system. The saline or corn oil test article extract was clear, colorless liquid or clear, yellow liquid, respectively.

Micronucleus Assay

The results of the micronucleus assay are presented in Tables 1 through 4.

For the micronucleus test, male and female mice were dosed either with saline or corn oil extracts of the test article, saline or corn oil extraction blanks or the positive control article (CP). No mortality or clinical signs were observed during the course of the study. All mice appeared normal throughout the observation period (Table 1).

The incidence of micronucleated polychromatic erythrocytes per 2000 polychromatic erythrocytes scored and the proportion of polychromatic erythrocytes per total erythrocytes are summarized and presented for each treatment group by sacrifice time in Table 2. Individual animal 24 hours post-dose data and 48 post-dose data are presented in Tables 3 and 4, respectively for both, the saline and corn oil test article extracts. No appreciable reductions in the ratio of polychromatic erythrocytes to total erythrocytes were observed in test article extract-treated groups relative to the respective extraction blank (negative) controls suggesting that test article extracts did not inhibit erythropoiesis. The number of micronucleated polychromatic erythrocytes per 10000 polychromatic erythrocytes in test article extract-treated groups was not statistically increased relative to the respective negative controls in either male or female mice, regardless of bone marrow collection time ($p > 0.05$, Kastenbaum-Bowman Tables).

All criteria specified in the protocol were met. CP induced a significant increase in micronucleated polychromatic erythrocytes in both male and female mice ($p \leq 0.05$, Kastenbaum-Bowman Tables). Additionally, the negative and positive controls were consistent with the historical control data, indicating that there was no problem with the test system or the quality of the test.

CONCLUSION

The results of the assay indicate that under the conditions described in this report, the saline and corn oil extracts of the test article did not induce a significant increase in micronucleated polychromatic erythrocytes in either male or female ICR mice. Therefore, saline and corn oil extracts of 300cc Siltex Moderate Profile Gel-filled Mammary Prosthesis were concluded to be negative in the micronucleus test.

REFERENCES

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Table 1

Clinical Signs Following Dose Administration of
Saline and Corn oil Extracts of 300cc Siltex Moderate Profile Gel-filled Mammary Prosthesis in ICR Mice

| Treatment (20 mL/kg) | Observation | Number of Animals With Clinical Signs/Total Number of Animals Dosed | | Number of Animals Died/Total Number of Animals Dosed | |
|---------------------------|-------------|---|---------|--|---------|
| | | Males | Females | Males | Females |
| Saline Extraction Blank | Normal | 10/10 | 10/10 | 0/10 | 0/10 |
| Saline TA Extract | Normal | 10/10 | 10/10 | 0/10 | 0/10 |
| Corn Oil Extraction Blank | Normal | 10/10 | 10/10 | 0/10 | 0/10 |
| Corn Oil TA Extract | Normal | 10/10 | 10/10 | 0/10 | 0/10 |
| CP: 50 mg/kg | Normal | 5/5 | 5/5 | 0/5 | 0/5 |

TA = 300cc Siltex Moderate Profile Gel-filled Mammary Prosthesis
CP = Cyclophosphamide monohydrate

Table 2

Summary of Bone Marrow Micronucleus Analysis
Following Dose Administration of Saline and Corn oil Extracts of
300cc Siltex Moderate Profile Gel-filled Mammary Prosthesis in ICR Mice

| Treatment (20 mL/kg) | Sex | Time (hr) | Number of Mice | PCE/Total Erythrocytes (Mean +/- SD) | Change from Control (%) | Micronucleated Polychromatic Erythrocytes | |
|-------------------------------|-----|--------------|-------------------|--|----------------------------|---|--|
| | | | | | | Number per 1000 PCEs (Mean +/- SD) | Number per PCEs Scored ¹ |
| Saline Extraction Blank | | | | | | | |
| | M | 24 | 5 | 0.468 ± 0.07 | -- | 0.3 ± 0.27 | 3 / 10000 |
| | F | 24 | 5 | 0.494 ± 0.07 | -- | 0.3 ± 0.27 | 3 / 10000 |
| Saline Test Article Extract | | | | | | | |
| | M | 24 | 5 | 0.456 ± 0.02 | -3 | 0.3 ± 0.27 | 3 / 10000 |
| | F | 24 | 5 | 0.453 ± 0.02 | -8 | 0.3 ± 0.27 | 3 / 10000 |
| CP** | | | | | | | |
| 50 mg/kg | M | 24 | 5 | 0.346 ± 0.02 | -26 | 20.4 ± 1.08 | *204 / 10000 |
| | F | 24 | 5 | 0.316 ± 0.02 | -36 | 23.2 ± 3.88 | *232 / 10000 |
| Saline Extraction Blank | | | | | | | |
| | M | 48 | 5 | 0.447 ± 0.03 | -- | 0.5 ± 0.35 | 5 / 10000 |
| | F | 48 | 5 | 0.488 ± 0.05 | -- | 0.5 ± 0.35 | 5 / 10000 |
| Saline Test Article Extract | | | | | | | |
| | M | 48 | 5 | 0.485 ± 0.03 | 9 | 0.7 ± 0.27 | 7 / 10000 |
| | F | 48 | 5 | 0.451 ± 0.03 | -8 | 0.3 ± 0.27 | 3 / 10000 |
| Corn Oil Extraction Blank | | | | | | | |
| | M | 24 | 5 | 0.438 ± 0.02 | -- | 0.2 ± 0.27 | 2 / 10000 |
| | F | 24 | 5 | 0.451 ± 0.04 | -- | 0.5 ± 0.35 | 5 / 10000 |
| Corn Oil Test Article Extract | | | | | | | |
| | M | 24 | 5 | 0.439 ± 0.02 | 0 | 0.4 ± 0.22 | 4 / 10000 |
| | F | 24 | 5 | 0.470 ± 0.03 | 4 | 0.6 ± 0.22 | 6 / 10000 |
| CP** | | | | | | | |
| 50 mg/kg | M | 24 | 5 | 0.346 ± 0.02 | -21 | 20.4 ± 1.08 | *204 / 10000 |
| | F | 24 | 5 | 0.316 ± 0.02 | -30 | 23.2 ± 3.88 | *232 / 10000 |
| Corn Oil Extraction Blank | | | | | | | |
| | M | 48 | 5 | 0.480 ± 0.07 | -- | 0.5 ± 0.00 | 5 / 10000 |
| | F | 48 | 5 | 0.457 ± 0.04 | -- | 0.6 ± 0.22 | 6 / 10000 |
| Corn Oil Test Article Extract | | | | | | | |
| | M | 48 | 5 | 0.479 ± 0.04 | 0 | 0.5 ± 0.00 | 5 / 10000 |
| | F | 48 | 5 | 0.438 ± 0.04 | -4 | 0.7 ± 0.45 | 7 / 10000 |

¹*Statistically significant, p<0.05 (Kastenbaum-Bowman Tables).

**Only one positive control group was part of the study. 24 hrs post-dose CP data were compared with both the saline and corn oil extraction blanks.

Table 3

Induction of Micronucleated Polychromatic Erythrocytes in Bone Marrow Cells
Collected 24 Hours Following Dose Administration of Saline and Corn oil Extracts of
300cc Siltex Moderate Profile Gel-filled Mammary Prosthesis in ICR Mice

| Treatment (20 mL/kg) | Sex | Animal Number | PCE/Total Erythrocytes | Micronucleated PCE (Number/PCE scored) |
|-------------------------------|-----|---------------|------------------------|--|
| Saline Extraction Blank | M | 101 | 0.430 | 0 / 2000 |
| | | 102 | 0.416 | 1 / 2000 |
| | | 103 | 0.568 | 0 / 2000 |
| | | 104 | 0.511 | 1 / 2000 |
| | | 105 | 0.417 | 1 / 2000 |
| | F | 106 | 0.416 | 1 / 2000 |
| | | 107 | 0.425 | 1 / 2000 |
| | | 108 | 0.571 | 1 / 2000 |
| | | 109 | 0.534 | 0 / 2000 |
| | | 110 | 0.523 | 0 / 2000 |
| Saline Test Article Extract | M | 111 | 0.467 | 0 / 2000 |
| | | 112 | 0.480 | 1 / 2000 |
| | | 113 | 0.444 | 1 / 2000 |
| | | 114 | 0.423 | 1 / 2000 |
| | | 115 | 0.464 | 0 / 2000 |
| | F | 116 | 0.453 | 1 / 2000 |
| | | 117 | 0.448 | 1 / 2000 |
| | | 118 | 0.446 | 0 / 2000 |
| | | 119 | 0.490 | 1 / 2000 |
| | | 120 | 0.428 | 0 / 2000 |
| CP 50 mg/kg | M | 141 | 0.370 | 44 / 2000 |
| | | 142 | 0.367 | 41 / 2000 |
| | | 143 | 0.348 | 38 / 2000 |
| | | 144 | 0.326 | 40 / 2000 |
| | | 145 | 0.317 | 41 / 2000 |
| | F | 146 | 0.297 | 55 / 2000 |
| | | 147 | 0.312 | 50 / 2000 |
| | | 148 | 0.341 | 34 / 2000 |
| | | 149 | 0.321 | 46 / 2000 |
| | | 150 | 0.307 | 47 / 2000 |
| Corn Oil Extraction Blank | M | 121 | 0.447 | 0 / 2000 |
| | | 122 | 0.425 | 1 / 2000 |
| | | 123 | 0.460 | 1 / 2000 |
| | | 124 | 0.421 | 0 / 2000 |
| | | 125 | 0.435 | 0 / 2000 |
| | F | 126 | 0.419 | 0 / 2000 |
| | | 127 | 0.518 | 2 / 2000 |
| | | 128 | 0.431 | 1 / 2000 |
| | | 129 | 0.441 | 1 / 2000 |
| | | 130 | 0.444 | 1 / 2000 |
| Corn Oil Test Article Extract | M | 131 | 0.463 | 1 / 2000 |
| | | 132 | 0.457 | 1 / 2000 |
| | | 133 | 0.414 | 1 / 2000 |
| | | 134 | 0.411 | 0 / 2000 |
| | | 135 | 0.451 | 1 / 2000 |
| | F | 136 | 0.466 | 1 / 2000 |
| | | 137 | 0.456 | 1 / 2000 |
| | | 138 | 0.513 | 1 / 2000 |
| | | 139 | 0.428 | 1 / 2000 |
| | | 140 | 0.486 | 2 / 2000 |

Table 4

Induction of Micronucleated Polychromatic Erythrocytes in Bone Marrow Cells
Collected 48 Hours After a Single Dose of Saline and Corn Oil Extracts of
300cc Siltex Moderate Profile Gel-filled Mammary Prosthesis in ICR Mice

| Treatment (20 mL/kg) | Sex | Animal Number | PCE/Total Erythrocytes | Micronucleated PCE (Number/PCE scored) |
|-------------------------------|-----|---------------|------------------------|--|
| Saline Extraction Blank | M | 151 | 0.463 | 2 / 2000 |
| | | 152 | 0.433 | 1 / 2000 |
| | | 153 | 0.488 | 1 / 2000 |
| | | 154 | 0.419 | 0 / 2000 |
| | | 155 | 0.434 | 1 / 2000 |
| | F | 156 | 0.553 | 1 / 2000 |
| | | 157 | 0.431 | 0 / 2000 |
| | | 158 | 0.522 | 1 / 2000 |
| | | 159 | 0.468 | 1 / 2000 |
| | | 160 | 0.466 | 2 / 2000 |
| Saline Test Article Extract | M | 161 | 0.467 | 1 / 2000 |
| | | 162 | 0.488 | 2 / 2000 |
| | | 163 | 0.475 | 1 / 2000 |
| | | 164 | 0.540 | 1 / 2000 |
| | | 165 | 0.454 | 2 / 2000 |
| | F | 166 | 0.456 | 1 / 2000 |
| | | 167 | 0.405 | 1 / 2000 |
| | | 168 | 0.468 | 0 / 2000 |
| | | 169 | 0.470 | 0 / 2000 |
| | | 170 | 0.454 | 1 / 2000 |
| Corn Oil Extraction Blank | M | 171 | 0.400 | 1 / 2000 |
| | | 172 | 0.459 | 1 / 2000 |
| | | 173 | 0.548 | 1 / 2000 |
| | | 174 | 0.560 | 1 / 2000 |
| | | 175 | 0.431 | 1 / 2000 |
| | F | 176 | 0.418 | 1 / 2000 |
| | | 177 | 0.472 | 1 / 2000 |
| | | 178 | 0.419 | 2 / 2000 |
| | | 179 | 0.464 | 1 / 2000 |
| | | 180 | 0.512 | 1 / 2000 |
| Corn Oil Test Article Extract | M | 181 | 0.527 | 1 / 2000 |
| | | 182 | 0.414 | 1 / 2000 |
| | | 183 | 0.492 | 1 / 2000 |
| | | 184 | 0.488 | 1 / 2000 |
| | | 185 | 0.473 | 1 / 2000 |
| | F | 186 | 0.413 | 1 / 2000 |
| | | 187 | 0.409 | 2 / 2000 |
| | | 188 | 0.496 | 2 / 2000 |
| | | 189 | 0.421 | 2 / 2000 |
| | | 190 | 0.451 | 0 / 2000 |

000155

APPENDIX I:

Mouse Micronucleus Test Historical Control Data

000156

Mouse Micronucleus Test Historical Control Data

2000-2002

Negative Control¹

| Parameter | Ratio of PCE/Total Erythrocytes | | MPCE/1000 PCE Scored/Animal | | MPCE/5000 PCE Scored/Group | |
|--------------------|---------------------------------|-------------|-----------------------------|-------------|----------------------------|-------------|
| | Males | Females | Males | Females | Males | Females |
| Mean ³ | 0.49 | 0.50 | 0.36 | 0.42 | 1.80 | 2.09 |
| Standard Deviation | 0.06 | 0.06 | 0.33 | 0.36 | 0.89 | 1.05 |
| Range ⁴ | 0.23 - 0.71 | 0.31 - 0.71 | 0.00 - 2.50 | 0.00 - 2.00 | 0.00 - 4.50 | 0.00 - 6.50 |

Positive Control²

| Parameter | Ratio of PCE/Total Erythrocytes | | MPCE/1000 PCE Scored/Animal | | MPCE/5000 PCE Scored/Group | |
|--------------------|---------------------------------|-------------|-----------------------------|--------------|----------------------------|----------------|
| | Males | Females | Males | Females | Males | Females |
| Mean ³ | 0.39 | 0.39 | 23.96 | 23.95 | 119.80 | 119.73 |
| Standard Deviation | 0.09 | 0.09 | 8.61 | 8.44 | 33.15 | 32.59 |
| Range ⁴ | 0.13 - 0.75 | 0.17 - 0.76 | 5.50 - 79.00 | 6.00 - 67.00 | 46.00 - 250.50 | 64.00 - 246.00 |

¹Negative control articles: all vehicles, solvents or carriers; Route of administration: intravenous (IV), intraperitoneal (IP) or oral gavage (PO). Bone marrow collection time: 24 and 48 hours post-dose.

²Positive control: Cyclophosphamide monohydrate (CP); Doses: 30 to 60 mg/kg; Route of administration: IV, IP or PO. Bone marrow collection time: 24 hours post-dose.

³ Average of the PCE ratio observed out of 1000 erythrocytes scored per animal for the total number of animals used during 2000-2002; average of the number of MPCE per 1000 PCE for the total number of animals used in 2000-2002; average of number of MPCE/per group (containing 5 animals per group) for total number of groups used in 2000-2002.

⁴ Minimum and maximum range of PCE ratio observed out of 1000 erythrocytes scored per animal; the minimum and maximum range of MPCE observed out of 1000 PCE for the total number of animals used in 2000-2002 and the minimum and maximum range of MPCE observed out of 5000 PCE for the total number of groups used in 2000-2002.

APPENDIX II:
Animal Body Weight Data

000158

Animal Body Weights at Dose Administration

| Treatment at 20 mL/kg | | Animal Number | Weight (g) |
|-----------------------|--------------|---------------|------------|
| Group | 1A | 101 | 30.3 |
| Sex | Male | 102 | 30.7 |
| Dose | Saline | 103 | 33.0 |
| | Extraction | 104 | 34.5 |
| | Blank | 105 | 34.4 |
| | | Mean | 32.6 |
| | | S.D. | 1.99 |
| | | N | 5 |
| Group | 1A | 106 | 28.1 |
| Sex | Female | 107 | 27.8 |
| Dose | Saline | 108 | 26.6 |
| | Extraction | 109 | 28.0 |
| | Blank | 110 | 29.6 |
| | | Mean | 28.0 |
| | | S.D. | 1.07 |
| | | N | 5 |
| Group | 2A | 111 | 32.1 |
| Sex | Male | 112 | 31.9 |
| Dose | Saline | 113 | 33.5 |
| | Test Article | 114 | 33.5 |
| | Extract | 115 | 34.8 |
| | | Mean | 33.2 |
| | | S.D. | 1.19 |
| | | N | 5 |
| Group | 2A | 116 | 26.7 |
| Sex | Female | 117 | 28.4 |
| Dose | Saline | 118 | 30.1 |
| | Test Article | 119 | 27.9 |
| | Extract | 120 | 29.7 |
| | | Mean | 28.6 |
| | | S.D. | 1.38 |
| | | N | 5 |

Animal Body Weights at Dose Administration: Micronucleus Assay (Cont'd)

| Treatment at 20 mL/kg | | Animal Number | Weight (g) |
|-----------------------|--------------|---------------|------------|
| Group | 3A | 121 | 31.9 |
| Sex | Male | 122 | 32.3 |
| Dose | Corn oil | 123 | 32.1 |
| | Extraction | 124 | 33.7 |
| | Blank | 125 | 36.4 |
| | | Mean | 33.3 |
| | | S.D. | 1.88 |
| | | N | 5 |
| Group | 3A | 126 | 27.6 |
| Sex | Female | 127 | 29.0 |
| Dose | Corn oil | 128 | 27.7 |
| | Extraction | 129 | 30.3 |
| | Blank | 130 | 30.5 |
| | | Mean | 29.0 |
| | | S.D. | 1.38 |
| | | N | 5 |
| Group | 4A | 131 | 32.7 |
| Sex | Male | 132 | 32.1 |
| Dose | Corn oil | 133 | 31.9 |
| | Test Article | 134 | 33.4 |
| | Extract | 135 | 35.6 |
| | | Mean | 33.1 |
| | | S.D. | 1.49 |
| | | N | 5 |
| Group | 4A | 136 | 26.3 |
| Sex | Female | 137 | 27.2 |
| Dose | Corn oil | 138 | 27.4 |
| | Test Article | 139 | 27.2 |
| | Extract | 140 | 31.2 |
| | | Mean | 27.9 |
| | | S.D. | 1.92 |
| | | N | 5 |

000160

Animal Body Weights at Dose Administration: Micronucleus Assay (Cont'd)

| Treatment at 20 mL/kg | | Animal Number | Weight (g) |
|-----------------------|-------------|---------------|------------|
| Group | 5 | 141 | 31.1 |
| Sex | Male | 142 | 32.2 |
| Dose | CP 50 mg/kg | 143 | 33.3 |
| | | 144 | 33.3 |
| | | 145 | 35.0 |
| | | Mean | 33.0 |
| | | S.D. | 1.45 |
| | | N | 5 |
| Group | 5 | 146 | 27.9 |
| Sex | Female | 147 | 27.5 |
| Dose | CP 50 mg/kg | 148 | 30.2 |
| | | 149 | 28.5 |
| | | 150 | 31.5 |
| | | Mean | 29.1 |
| | | S.D. | 1.68 |
| | | N | 5 |
| Group | 1B | 151 | 32.4 |
| Sex | Male | 152 | 33.2 |
| Dose | Saline | 153 | 31.4 |
| | Extraction | 154 | 34.3 |
| | Blank | 155 | 34.0 |
| | | Mean | 33.1 |
| | | S.D. | 1.19 |
| | | N | 5 |
| Group | 1B | 156 | 25.9 |
| Sex | Female | 157 | 27.6 |
| Dose | Saline | 158 | 28.3 |
| | Extraction | 159 | 29.2 |
| | Blank | 160 | 27.6 |
| | | Mean | 27.7 |
| | | S.D. | 1.21 |

Animal Body Weights at Dose Administration: Micronucleus Assay (Cont'd)

| Treatment at 20 mL/kg | | Animal Number | Weight (g) |
|-----------------------|--------------|---------------|------------|
| Group | 2B | 161 | 32.4 |
| Sex | Male | 162 | 31.1 |
| Dose | Saline | 163 | 33.1 |
| | Test Article | 164 | 34.8 |
| | Extract | 165 | 34.6 |
| | | Mean | 33.2 |
| | | S.D. | 1.55 |
| | | N | 5 |
| Group | 2B | 166 | 26.4 |
| Sex | Female | 167 | 30.6 |
| Dose | Saline | 168 | 27.7 |
| | Test Article | 169 | 29.2 |
| | Extract | 170 | 28.6 |
| | | Mean | 28.5 |
| | | S.D. | 1.58 |
| | | N | 5 |
| Group | 3B | 171 | 31.5 |
| Sex | Male | 172 | 31.6 |
| Dose | Corn oil | 173 | 32.8 |
| | Extraction | 174 | 33.6 |
| | Blank | 175 | 35.9 |
| | | Mean | 33.1 |
| | | S.D. | 1.80 |
| | | N | 5 |
| Group | 3B | 176 | 26.6 |
| Sex | Female | 177 | 27.9 |
| Dose | Corn oil | 178 | 29.8 |
| | Extraction | 179 | 32.8 |
| | Blank | 180 | 29.0 |
| | | Mean | 29.2 |
| | | S.D. | 2.33 |
| | | N | 5 |

000162

Animal Body Weights at Dose Administration: Micronucleus Assay (Cont'd)

| Treatment at 20 mL/kg | | Animal Number | Weight (g) |
|-----------------------|--------------|---------------|------------|
| Group | 4B | 181 | 30.7 |
| Sex | Male | 182 | 33.9 |
| Dose | Corn oil | 183 | 32.6 |
| | Test Article | 184 | 33.4 |
| | Extract | 185 | 33.7 |
| | | Mean | 32.9 |
| | | S.D. | 1.30 |
| | | N | 5 |
| Group | 4B | 186 | 27.4 |
| Sex | Female | 187 | 28.5 |
| Dose | Corn oil | 188 | 28.0 |
| | Test Article | 189 | 29.0 |
| | Extract | 190 | 29.2 |
| | | Mean | 28.4 |
| | | S.D. | 0.74 |
| | | N | 5 |

000163

APPENDIX III:

Study Protocol

000164

Received by RA/OA 03/19/03

QA 8054463
APPROVED

BioReliance Study Number: AA731P.123201.BTL

**Micronucleus Cytogenetic Assay in Mice Conducted with
Test Article Extracts**

1.0 PURPOSE

The purpose of this study is to evaluate the clastogenic potential of saline and corn oil extracts of the test article as measured by its ability to induce micronucleated polychromatic erythrocytes in mouse bone marrow.

2.0 SPONSOR

- 2.1 Sponsor Name: **Mentor Corporation**
- 2.2 Address: **201 Mentor Drive
Santa Barbara, CA 93111**
- 2.3 Representative: **Philip Yang
Phone: 805-879-6427
Fax: 805-879-6014
Email: pyang@mentorcorp.com**
- 2.4 Sponsor Project #: **(NONE)**

3.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

- 3.1 Test Article: **300cc Siltex Moderate Profile Gel-filled Mammary Prosthesis (Cat. #354-3007, Lot #257949) made with [REDACTED] materials, [REDACTED] patches, new calender, [REDACTED] mandrels, and [REDACTED] sterilization. The sterile device was cut open to extract both the gel and the shell.**
- 3.2 Controls: **Negative: Saline and corn oil extraction blanks
Positive: Cyclophosphamide (CP)**

3.3 Determination of Strength, Purity, etc.

Unless alternate arrangements are made, the testing facility at BioReliance will not perform analysis of the dosing solutions. The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test article and the stability and strength of the dosing solutions.

3.4 Test Article Retention Sample

The retention of a reserve sample of the test article will be the responsibility of the Sponsor.

[REDACTED]

[REDACTED]

4.0 TESTING FACILITY AND KEY PERSONNEL

- 4.1 Name: Toxicology Testing Facility
BioReliance
- 4.2 Address: 9630 Medical Center Drive
Rockville, MD 20850
- 4.3 Study Director: Ramadevi Gudi, Ph.D.
Phone: (301) 610-2169
Fax: (301) 738-2362
E-mail: rgudi@bioreliance.com

5.0 TEST SCHEDULE

- 5.1 Proposed Experimental Initiation Date: *April 15, 2003*
- 5.2 Proposed Experimental Completion Date: *May 5, 2003*
- 5.3 Proposed Report Date: *May 16, 2003*

6.0 TEST SYSTEM

Closed-colony, random-bred rodents are acceptable models for mutagenicity studies. ICR mice were selected because of the availability of historical control data.

- 6.1 Source: Harlan Sprague Dawley, Inc.
Frederick, MD or
Charles River Breeding Laboratories
Kingston, NY or Raleigh, NC
or other approved alternates
- 6.2 Age at initiation of study: 6-8 weeks

7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

Following the administration of one concentration each of saline or corn oil test article extracts, positive or negative (extract blank) controls to male and female mice, bone marrow cells will be collected at 24 and 48 hours and examined for the presence of micronucleated polychromatic erythrocytes. The clastogenic potential of the test article extract will be measured by its ability to increase micronucleated polychromatic erythrocytes in treated animals as compared to negative control animals. The study design will be as follows:

| | Number per Sex to be Sacrificed After Dose Administration | |
|-------------------------------|--|-------|
| | 24 Hr | 48 Hr |
| Saline Extraction Blank | 5 | 5 |
| Corn Oil Extraction Blank | 5 | 5 |
| Saline Test Article Extract | 5 | 5 |
| Corn Oil Test Article Extract | 5 | 5 |
| Positive Control (CP) | 5 | |

7.1 Extract Preparation

The Sponsor has specified that extracts of the test article will be prepared using saline and corn oil according to the following procedures (USP (XXIV), <88> Biological Reactivity Test, *In Vivo*, p. 1832-1836.). The test article will be extracted with 20 mL extraction medium for each of the following specimen sizes: 120 cm² total surface area (both sides) for specimens 0.5 mm or less in thickness or 60 cm² total surface area (both sides) for specimens >0.5 mm in thickness. When surface area cannot be readily determined due to the configuration of the specimen, an extraction ratio based on 0.1 g of elastomer or 0.2 g of plastic or other polymers for every 1 mL of extracting fluid will be used. The test article will be placed in the extraction vessel and immersed in the required volume of extraction medium. The test article may be cut into small pieces to facilitate total immersion in the extraction medium. The headspace of the extraction vessel will be purged with nitrogen gas and the vessel capped tightly. The extraction mixture will be shaken for 72±2 hours at 50±2°C. At the end of the extraction period, the vessel will be cooled to room temperature and shaken vigorously for several minutes. The extract will be aseptically decanted into a dry, sterile vessel, and stored at room temperature. Saline and corn oil extraction blanks will be prepared in the same manner, without the addition of test article. All extracts and extraction blanks will be used within 24 hours of preparation.

7.2 Dose Selection

In consultation with the Sponsor, there will be no preliminary toxicity tests performed with either the saline or corn oil extract of the device. The animals will be dosed with each test article extract or each extract blank at 20 mL extract/kg body weight based on the maximum allowable dosing volume. In the event of mortality in excess of 50% at 20 mL extract/kg, the Sponsor will be consulted concerning the need for toxicity testing and the possible need for a multiple dose level micronucleus assay. This additional testing would require a separate protocol and testing agreement.

7.3 Route and Frequency of Administration

Animals will be dosed by intraperitoneal (IP) injection. IP injection was selected to maximize delivery of the test article to the target system. IP injection is an

acceptable method for administration of test article concentrations to laboratory animals. Animals will receive the test article extracts and controls as a single administration.

7.4 Controls

7.4.1 Negative control

The saline and corn oil extraction blanks will be used as the negative controls.

7.4.2 Positive control

Cyclophosphamide (CP) will be administered as the positive control at a dose of 30-60 mg/kg. CP will be administered by the same route as the test article extract.

7.5 Animal Receipt and Quarantine

Virus antibody-free (VAF) mice will be quarantined for no less than 5 days prior to dose administration. The animals will be observed each working day for signs of illness, unusual food and water consumption, and other general conditions of poor health. All animals will be judged to be healthy prior to utilization in the study.

7.6 Animal Care

Animals will be housed in an AAALAC-accredited facility with a controlled environment of $50 \pm 20\%$ relative humidity and $72 \pm 3^\circ\text{F}$ temperature with a 12 hour light/dark cycle. Mice of the same sex will be housed up to five per cage in plastic autoclavable cages. Heat-treated hardwood chips will be used for bedding. Animals will have free access to a certified laboratory rodent chow which has been analyzed for environmental contaminants and to tap water.

7.7 Randomization

The animals will be assigned to nine groups of five males and five females using a randomization procedure which is based on equalization of group mean body weights. Additional animals may be designated and dosed as replacement animals in the test article extract high dose group to be used in the event of mortality prior to the scheduled sacrifice. This will be done at the discretion of the Study Director after evaluation of the toxicity data. Each animal will be given a sequential number and identified by an ear tag.

7.8 Dose Administration

The test article extracts, the extraction blank controls and the positive control (CP) will be given as single administrations. The volume of administration will be 20 mL/kg body weight. All mice in the experimental groups will be weighed and the dose volume will be based on individual body weight.

7.9 Bone Marrow Collection

Twenty-four and 48 hours after dose administration, five animals per sex per test article extract-treated and negative control groups will be sacrificed by carbon dioxide asphyxiation. The positive control group will be sacrificed 24 hours after dose administration. Immediately following sacrifice, the femurs will be exposed, cut just above the knee and the bone marrow will be aspirated into a syringe containing fetal bovine serum. The bone marrow cells will be transferred to a capped centrifuge tube containing approximately 1 ml fetal bovine serum.

The bone marrow cells will be pelleted by centrifugation and the supernatant will be drawn off, leaving a small amount of fetal bovine serum with the remaining cell pellet. The cells will be resuspended by aspiration with a capillary pipette and a small drop of the bone marrow suspension will be spread onto a clean glass slide. Each slide will be identified by the experiment and animal number. At least two slides will be prepared from each animal, air dried, fixed by dipping in methanol, stained with May-Gruenwald-Giemsa stain and permanently mounted.

7.10 Scoring for Micronuclei

Slides will be coded using a random number table by an individual not involved with the scoring process. Using medium magnification, an area of acceptable quality will be selected such that the cells are well spread and stained. Using oil immersion, 2000 polychromatic erythrocytes will be scored for the presence of micronuclei. The number of micronucleated normocytes in the field of 1000 polychromatic erythrocytes will also be enumerated. The proportion of polychromatic erythrocytes to total erythrocytes will also be recorded per 1000 erythrocytes. The proportion of polychromatic erythrocytes to total erythrocytes in test article extract-treated animals should not be less than 20% of the control value.

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

The mean incidence of micronucleated polychromatic erythrocytes must not exceed 5/1000 polychromatic erythrocytes (0.5%) in the negative (extraction blank) control. The incidence of micronucleated polychromatic erythrocytes in the positive control group must be significantly increased relative to the negative control ($p \leq 0.05$, Kastenbaum-Bowman Tables).

9.0 EVALUATION OF TEST RESULTS

The incidence of micronucleated polychromatic erythrocytes per 2000 polychromatic erythrocytes will be presented for each animal and treatment group. Statistical significance will be determined using the Kastenbaum-Bowman Tables which are based on the binomial distribution. All analyses will be performed separately for each sex.

In order to quantify the test article extract effect on erythropoiesis, as an indicator of bone marrow toxicity, the proportion of polychromatic erythrocytes to total erythrocytes will be presented for each animal and treatment group.

All conclusions will be based on sound scientific judgement; however, as a guide to interpretation of the data, the test article extract will be considered to induce a positive response if a statistically significant increase in micronucleated polychromatic erythrocytes is observed relative to the negative control ($p \leq 0.05$, Kastenbaum-Bowman Tables) at any sampling time. However, values that are statistically significant but do not exceed the range of historical negative or vehicle controls may be judged as not biologically significant. The test article extract will be judged negative if no statistically significant increase in micronucleated polychromatic erythrocytes above the concurrent negative control values are observed at any sampling time.

10.0 REPORT

A report of the results of this study will be prepared by the Testing Laboratory and will accurately describe all methods used for generation and analysis of the data.

The report will include:

- Test article: identification and CAS no., if known; physical nature and purity, if known; physicochemical properties relevant to the conduct of the study, if known; stability of the test article, if known.
- Extraction Media: justification for choice of extraction media; details on test article extraction
- Test animals: species and strain of animals used, age of animals, number of animals for each sex in experimental and control groups.
- Test Conditions: detailed description of and rationale for device extraction; details of the administration of test article extract; rationale for route of administration; methods for verifying that the test article extract reached the general circulation or target tissue, if applicable; details of food and water quality; description of treatment and sampling schedules; treatment procedures; doses; toxicity data; negative (extract blank) and positive control data; method of slide preparation; methods for measurement of toxicity; criteria for scoring micronucleated immature erythrocytes; number of cells analyzed per animal; criteria for considering study as positive, negative or equivocal
- Results: signs of toxicity; proportion of polychromatic erythrocytes among total erythrocytes; number of micronucleated polychromatic erythrocytes per animal; mean \pm standard deviation of micronucleated polychromatic erythrocytes per group; dose-response relationship, where possible; statistical analyses; concurrent negative control data; historical negative control data with ranges, means and standard deviations; concurrent positive control data
- Discussion of results
- Conclusion.

11.0 RECORDS AND ARCHIVES

All raw data, protocol, and a copy of all reports will be maintained according to Standard Operating Procedure OPOP3040 by the BioReliance RAQA unit headquartered at BioReliance, 14920 Broschart Road, Rockville, MD 20850. Per this SOP, paper records will be retained for at least three years after which time the Sponsor will be contacted for a decision as to the final disposition of the materials. All study materials returned to the Sponsor or destroyed will first be copied and the copy will be retained in the BioReliance archives for a minimum of 10 years.

12.0 REGULATORY REQUIREMENTS/GOOD LABORATORY PRACTICE

This protocol has been written to comply with EEC Directive 79/831 Annex V, B.12 Mutagenicity (Micronucleus Test), June, 1989; OECD Guideline 474 (Genetic Toxicology: Micronucleus Test), Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, published by OECD, Paris, February 1998; EPA Health Effects Testing Guidelines, Subpart 870.5395 (In Vivo Mammalian Bone Marrow Cytogenetics Tests: Micronucleus assay), August 1998; and Notification No. 118 of the Pharmaceutical Affairs Bureau, Ministry of Health and Welfare, Japan, February 15, 1984.

This study will be performed in compliance with the provisions of the Good Laboratory Practice Regulations for Nonclinical Laboratory Studies (GLPs). The protocol, an in-process phase, the raw data, and report(s) will be audited per the Standard Operating Procedures (SOPs) of BioReliance by the Quality Assurance Unit of BioReliance for compliance with GLPs, the SOPs of BioReliance and the study protocol. The in-process inspection will be performed to audit the critical assay procedures and systems supporting the assay. A signed QA statement will be included in the final report. This statement will list the system phases inspected during the previous quarter or the study-specific phases, the dates of each inspection, and the dates the results of each inspection were reported to the Study Director and the Study Director's management. In addition, a signed GLP compliance statement will be included in the final report. This statement will cite the GLP guideline(s) with which the study is compliant and any exceptions to this compliance, if applicable, including the omission of characterization or stability analyses of the test or control articles or their mixtures.

Will this study be submitted to a regulatory agency? Yes

If so to which agency or agencies? USFDA, Japan, Australia, EU

Unless arrangements are made to the contrary, unused dosing solutions will be disposed of following administration to the test system and all residual test article extract will be disposed of following finalization of the report.

13.0 REFERENCES

Hayashi, M., R.R. Tice, J.T. Macgregor, D. Anderson, D.H. Blakey, M. Dirsch-Volders, F.G. Oleson Jr., F. Pacchierotti, F. Romagna, H. Shimada, S. Sutou and B. Vannier. 1994. *In vivo* rodent erythrocyte micronucleus assay. *Mutation Res.* 312: 293-304.

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International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use. Genotoxicity: A Standard Battery for Genotoxicity Testing of Pharmaceuticals. S2B document recommended for adoption at step 4 of the ICH process on July 16, 1997. *Federal Register* 62:16026-16030, November 21, 1997.

Kastenbaum, M.A. and Bowman, K.O.. 1970. Tables for determining the statistical significance of mutation frequencies. *Mutation Res.* 9:527-549.

Mavourmin, K.H., D.H. Blakey, M.C. Cimino, M.F. Salamone and J.A. Heddle. 1990. The *in vivo* micronucleus assay in mammalian bone marrow and peripheral blood. A report of the U.S. Environmental Protection Agency Gene-Tox Program. *Mutation Res.* 239:29-80.

OECD Guideline for the Testing of Chemicals, OECD Guideline 474 (Genetic Toxicology: Mammalian Erythrocytes Micronucleus Test), Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, published by OECD, Paris, February 1998.

USP (XXIV), <88> Biological Reactivity Test, *In Vivo*, p. 1832-1836.

14.0 APPROVAL

Philip S. Yang
Sponsor Representative

3/26/03
Date

PHILIP S. YANG
(Print or Type Name)

Ramadevi Audi
BioReliance Study Director

03 Apr 2003
Date

[Signature]
BioReliance Study Management

03 Apr 2003
Date



FINAL REPORT

Study Title

***In Vitro* Mammalian Cell Gene Mutation Test
Conducted with Test Article Extracts**

Test Article

**300cc Siltex Moderate Profile Gel-filled Mammary Prosthesis
(Catalog # 354-3007, Lot # 257949)**

Authors

**Richard H. C. San, Ph.D.
Jane J. Clarke, M.S.**

Study Completion Date

10 June 2003

Testing Facility



BioReliance Study Number

AA73XP.702201.BTL

Sponsor

**Mentor Corporation
201 Mentor Drive
Santa Barbara, CA 93111**



STATEMENT OF COMPLIANCE

Study AA73XP.702201.BTL was conducted in compliance with the US FDA Good Laboratory Practice Regulations as published in 21 CFR 58, the US EPA GLP Standards 40 CFR 160, and 40 CFR 792, the UK GLP Compliance Regulations, the Japanese GLP Regulations, and the OECD Principles of Good Laboratory Practice in all material aspects with the following exceptions:

The identity, strength, purity, and composition or other characteristics to define the test article and the stability of the test article have not been determined by the testing facility or the Sponsor.

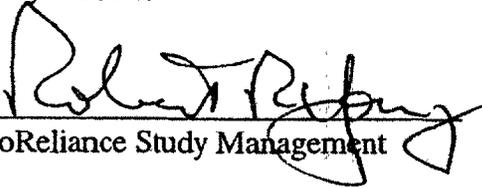
Analyses to determine the uniformity or concentration of the test mixtures and their stability were not performed by the testing facility or the Sponsor.



Richard H. C. San, Ph.D.
Study Director

10 Jun 2003

Date



BioReliance Study Management

11 June 2003

Date

Study Title: *In Vitro* Mammalian Gene Mutation Test Conducted with Test Article Extracts

Study Number: AA73XP.702201.BTL

Study Director: Richard H. C. San, Ph.D.

This study has been divided into a series of in-process phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment records, etc., are examined in order to assure that the study is performed in accordance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR 58), the U.S. EPA GLPs (40 CFR 792 and 40 CFR 160), the UK GLP Regulations, the Japanese GLP Standard, and the OECD Principles of Good Laboratory Practice and to assure that the study is conducted according to the protocol and relevant Standard Operating Procedures.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

Inspect On 04-Apr-03 - 04-Apr-03 To Study Dir 04-Apr-03 To Mgmt 04-Apr-03
Phase Protocol Review

Inspect On 23-May-03 - 28-May-03 To Study Dir 28-May-03 To Mgmt 29-May-03
Phase Draft Report

Inspect On 11-Jun-03 - 11-Jun-03 To Study Dir 11-Jun-03 To Mgmt 11-Jun-03
Phase Draft to Final Report

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Becky D. Schreckengost
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QUALITY ASSURANCE

11 June 2003
DATE

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In Vitro Mammalian Cell Gene Mutation Test
Conducted with Test Article Extracts

FINAL REPORT

Sponsor: **Mentor Corporation
201 Mentor Drive
Santa Barbara, CA 93111**

Authorized Representative: **Philip Yang**

Testing Facility: 

Test Article ID.: **300cc Siltex Moderate Profile Gel-filled
Mammary Prosthesis (Catalog # 354-3007)**

Test Article Lot No.: **257949**

BioReliance Study No.: **AA73XP.702201.BTL**

Test Article Description: **white opaque gel**

Storage Conditions: **room temperature; protected from light**

Test Article Receipt/Login: **28 March 2003/31 March 2003 (Sample 1)
30 April 2003/01 May 2003 (Sample 2)**

Study Initiation: **03 April 2003**

Experimental Start: **15 April 2003**

Experimental Completion: **19 May 2003**

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SUMMARY

The test article, 300cc Siltex Moderate Profile Gel-filled Mammary Prosthesis, was extracted in both saline and ethanol and each extract was tested in the L5178Y/TK⁺ Mouse Lymphoma Mutagenesis assay. In the mutagenesis assay, the test article extracts were tested at 100 μ L/mL with the saline extract and 10 μ L/mL with the ethanol extract. The test article extracts were administered full strength (undiluted) using a 1.0 mL dose volume for the saline extract and 100 μ L for the ethanol extract; these dose levels were limited by the maximum concentration of extraction medium compatible with the test system.

In the mutagenesis assay, no treated cultures exhibited mutant frequencies that were at least 55 mutants per 10⁶ clonable cells over that of the extraction blank control. No visible precipitate was present in the treatment medium of any cultures. Toxicity in the cloned cultures, i.e., total growth of \leq 50% of the extraction blank control, was not observed.

The TFT colonies for the positive and extraction blank control cultures were sized according to diameter over a range from approximately 0.2 to 1.1 mm. The colony sizing for the MMS positive control yielded the expected increase in small colonies, verifying the adequacy of the methods used to detect small colony mutants.

Under the conditions of this study, saline and ethanol extracts of the test article, 300cc Siltex Moderate Profile Gel-filled Mammary Prosthesis, were concluded to be negative in the L5178Y/TK⁺ Mouse Lymphoma Mutagenesis Assay.

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PURPOSE

The purpose of this study was to evaluate the mutagenic potential of saline and ethanol test article extracts based on quantitation of forward mutations at the thymidine kinase locus of L5178Y mouse lymphoma cells.

CHARACTERIZATION OF TEST AND CONTROL ARTICLES

The test article, 300cc Siltex Moderate Profile Gel-filled Mammary Prosthesis, was received by BioReliance on 28 March 2003 (Sample 1) and 01 May 2003 (Sample 2) and was assigned the code number AA73XP. The test article was characterized by the Sponsor as a sterile device, made with all SiTech materials, laser marked patches, new calender, stainless steel mandrels, and 35 hour dry heat sterilization, which should be stored at ambient temperature. Upon receipt, the test article was described as a white opaque gel and was stored at room temperature, protected from light.

Saline and ethanol extracts of the test article were prepared according to the following procedures. The test article was extracted at a ratio of 1.0 mL extraction medium per 0.2 grams test article. The test article was placed in the extraction vessel and immersed in 1414.5 mL saline extraction medium or 1546 mL ethanol extraction medium. The test article was cut into small pieces to expose the gel and the shell and to facilitate total immersion in the extraction medium. The headspace of the extraction vessels was purged with nitrogen gas and the vessel capped tightly. The extraction mixture was shaken for 72 ± 2 hours at $50 \pm 2^\circ\text{C}$. At the end of the extraction period, the vessels were cooled to room temperature and shaken vigorously for several minutes. The extracts were aseptically decanted into dry, sterile vessels and stored at room temperature. Saline and ethanol extraction blanks were prepared in the same manner, without the addition of test article. Test article extracts and extraction blanks were used within 24 hours of preparation.

The test article extraction media and positive control articles have been characterized as per the Certificates of Analysis on file with the testing facility. The stability of the test article extraction media and positive control articles and their mixtures was demonstrated by acceptable results that met the criteria for a valid test.

Methyl methanesulfonate (MMS), CAS 66-27-3, lot # 15526AO, expiration date March 2005, supplied by Aldrich Chemical Company was used as the positive control for the non-activated test system at stock concentrations of 1000 and 2000 $\mu\text{g}/\text{mL}$. 7,12-Dimethyl-benz(a)anthracene (7,12-DMBA), CAS 57-97-6, lot # 78H1078, expiration date January 2004, supplied by Sigma Chemical Company was used at stock concentrations of 250 and 400 $\mu\text{g}/\text{mL}$ as the positive control for the S9-activated test system.

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MATERIALS AND METHODS

Test System

L5178Y cells, clone 3.7.2C, were obtained from Dr. Patricia Poorman-Allen, Glaxo Inc., Research Triangle Park, NC. Each lot of cryopreserved cells was tested using the agar culture and Hoechst staining procedures and found to be free of mycoplasma contamination. Prior to use in the assay, L5178Y cells were cleansed of spontaneous TK⁺ cells by culturing in a restrictive medium (Clive and Spector, 1975).

Metabolic Activation System

Aroclor 1254-induced rat liver S9 was used as the metabolic activation system. The S9 was prepared from male Sprague-Dawley rats induced with a single intraperitoneal injection of Aroclor-1254, 500 mg/kg, five days prior to sacrifice. The S9 was batch prepared and stored at $\leq -70^{\circ}\text{C}$ until used. Each bulk preparation of S9 was assayed for sterility and its ability to metabolize 2-aminoanthracene and 7,12-dimethyl-benz(a)anthracene to forms mutagenic to *Salmonella typhimurium* TA100.

Immediately prior to use, the S9 was mixed with the cofactors and Fischer's Medium for Leukemic Cells of Mice with 0.1% Pluronics (F₀P) to contain 250 μl S9, 6.0 mg nicotinamide adenine dinucleotide phosphate (NADP), 11.25 mg DL-isocitric acid and 750 μl F₀P/mL S9-activation mixture and kept on ice until used. The cofactor/F₀P mixture was filter sterilized and adjusted to pH 7.0 prior to the addition of S9.

Mutagenesis Assay

The mutagenesis assay was used to evaluate the mutagenic potential of the test article extracts. Duplicate cultures of L5178Y mouse lymphoma cells were exposed to one concentration of each test article extract (undiluted) and the appropriate extraction blanks in both the absence and presence of S9. Positive controls, with and without S9-activation, were tested concurrently.

Treatment of the Target Cells

The mutagenesis assay was performed according to a protocol described by Clive and Spector (1975). Treatment was carried out in conical tubes by combining 6×10^6 L5178Y/TK⁺ cells, F₀P medium or S9 activation mixture and the appropriate aliquot of test article extract (1.0 mL for the saline extract or 100 μl for the ethanol extract) in a total volume of 10 mL. One concentration of each test article extract was tested in duplicate. Two control tubes received extraction blank only and the positive controls were treated with 100 μl MMS (at final concentrations of 10 and 20 $\mu\text{g}/\text{mL}$ in treatment medium) and 100 μl 7,12-DMBA (at final concentrations of 2.5 and 4.0 $\mu\text{g}/\text{mL}$ in treatment medium). Treatment tubes were gassed with $5 \pm 1\%$ CO₂ in air, capped tightly, and incubated with mechanical mixing for 4 hours at $37 \pm 1^{\circ}\text{C}$. The preparation and addition of the test article extract dosing solutions were carried out under amber lighting and the cells were incubated in the dark during the 4-hour exposure period. After

the treatment period of 4 hours at $37\pm 1^\circ\text{C}$, the cells were washed twice with F_0P or $F_{10}P$ supplemented with 10% horse serum and 2 mM L-glutamine ($F_{10}P$). After the second wash, the cells were resuspended in $F_{10}P$, gassed with $5\pm 1\%$ CO_2 in air and placed on the roller drum apparatus at $37\pm 1^\circ\text{C}$.

Expression of the Mutant Phenotype

For expression of the mutant phenotype, the cultures were counted using an electronic cell counter and adjusted to 3×10^5 cells/mL at approximately 24 and 48 hours after treatment in 20 and 10 mL total volume, respectively. Cultures with less than 3×10^5 cells/mL were not adjusted.

For expression of the TK^- cells, cells were placed in cloning medium (C.M.) containing 0.23% dissolved granulated agar in F_0P plus 20% horse serum. Two flasks per culture to be cloned were labeled with the test article extract concentration, activation condition, and either TFT (trifluorothymidine, the selective agent) or V.C. (viable count). Each flask was prewarmed to $37\pm 1^\circ\text{C}$, filled with 100 mL C.M., and placed in an incubator shaker at $37\pm 1^\circ\text{C}$ until used. The cells were centrifuged at 1000 rpm for 10 minutes and the supernatant was decanted. The cells were then diluted in C.M. to concentrations of 3×10^6 cells/100 mL C.M. for the TFT flask and 600 cells/100 mL C.M. for the VC flask. After the dilution, 1.0 mL of stock solution of TFT was added to the TFT flask (final concentration of 3 $\mu\text{g}/\text{mL}$) and both this flask and the V.C. flask were placed on the shaker at 125 rpm and $37\pm 1^\circ\text{C}$. After 15 minutes, the flasks were removed and 33 mL of the cell suspension was pipetted into each of three appropriately labeled petri dishes. To accelerate the gelling process, the plates were placed in cold storage (approximately 4°C) for approximately 30 minutes. The plates were then incubated at $37\pm 1^\circ\text{C}$ in a humidified $5\pm 1\%$ CO_2 atmosphere for 10-14 days.

Scoring Procedures

After the incubation period, the V.C. plates were counted for the total number of colonies per plate and the total relative growth determined. The TFT colonies were then counted for each culture with $\geq 10\%$ total relative growth. The diameters of the TFT colonies for the positive and extraction blank controls and, in the case of a positive response, the test article-treated cultures were determined over a range of approximately 0.2 to 1.1 mm. The rationale for this procedure is as follows: Mutant L5178Y TK^- colonies exhibit a characteristic frequency distribution of colony sizes. The precise distribution of large and small TFT-resistant mutant colonies appears to be the characteristic mutagenic "finger-print" of carcinogens in the L5178Y TK^- system (Clive *et al.*, 1979; DeMarini *et al.*, 1989). Clive *et al.* (1979) and Hozier *et al.* (1981) have presented evidence to substantiate the hypothesis that the small colony variants carry chromosome aberrations associated with chromosome 11, the chromosome on which the TK locus is located in the mouse (Kozak and Ruddle, 1977). They suggested that large colony mutants received very localized damage, possibly in the form of a point mutation or small deletion within the TK locus, while small colony mutants received damage to collateral loci concordant with the loss of TK activity.

Evaluation of Results

The cytotoxic effects of each treatment condition were expressed relative to the extraction blank-treated control for suspension growth over two days post-treatment and for total growth (suspension growth corrected for plating efficiency at the time of selection). The mutant frequency (number of mutants per 10^6 surviving cells) was determined by dividing the average number of colonies in the three TFT plates by the average number of colonies in the three corresponding V.C. plates and multiplying by the dilution factor (2×10^{-4}) then multiplying by 10^6 . For simplicity, this is described as: (Average # TFT colonies / average # VC colonies) x 200 in the tables.

In evaluation of the data, increases in mutant frequencies which occurred only at highly toxic concentrations (i.e., less than 10% total growth) were not considered biologically relevant. All conclusions were based on sound scientific judgement; however, the following criteria are presented as a guide to interpretation of the data (Clive *et al.*, 1995):

- The result will be considered to induce a positive response if test article extract-treated cultures with 10% or greater total growth exhibit mutant frequencies ≥ 100 mutants per 10^6 clonable cells over the background level.
- A result will be considered equivocal if the mutant frequency in treated cultures is between 55 and 99 mutants per 10^6 clonable cells over the background level.
- Test articles producing fewer than 55 mutants per 10^6 clonable cells over the background level will be concluded to be negative.

Criteria for a Valid Test

The following criteria must be met for the mutagenesis assay to be considered valid:

Negative Controls:

The spontaneous mutant frequency of the extraction blank cultures must be within 20 to 100 TFT-resistant mutants per 10^6 surviving cells. The cloning efficiency of the extraction blank groups must be greater than 50%.

Positive Controls:

At least one concentration of each positive control must exhibit mutant frequencies of ≥ 100 mutants per 10^6 clonable cells over the background level. The colony size distribution for the MMS positive control must show an increase in both small and large colonies (Moore *et al.*, 1985; Aaron *et al.*, 1994).

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Archives

All raw data, protocol, and a copy of all reports will be maintained according to Standard Operating Procedure OPQP3040 by the BioReliance RAQA unit headquartered at: BioReliance, 14920 Broschart Road, Rockville, MD 20850. Per this SOP, paper records will be retained for at least three years after which time the Sponsor will be contacted for a decision as to the final disposition of the materials. All study materials returned to the Sponsor or destroyed will first be copied and the copy will be retained in the BioReliance archives for a minimum of 10 years.

Deviations

No known deviations from the protocol or assay method SOPs occurred during the conduct of this study.

RESULTS AND DISCUSSION

Mutagenesis Assay

Saline Extract: The results of the mutagenesis assay for the saline extract are presented in Tables 1 through 4. Colony size distributions for the positive and negative controls are presented in Figures 1 and 2. The colony sizing for the MMS positive control yielded the expected increase in small colonies, verifying the adequacy of the methods used to detect small colony mutants. In the non-activated system, cultures treated with a test article extract concentration of 100 $\mu\text{L}/\text{mL}$ were cloned and produced suspension growths of 93% and 89%. In the S9-activated system cultures treated with a test article extract concentration of 100 $\mu\text{L}/\text{mL}$ were cloned and produced suspension growths of 107% and 105%. No test article precipitate was observed in treatment medium.

No treated cultures exhibited mutant frequencies that were at least 55 mutants per 10^6 clonable cells over that of the extraction blank control. The total growths were 107% and 111% for the non-activated cultures and 106% and 116% for the S9-activated cultures at a test article extract concentration of 100 $\mu\text{L}/\text{mL}$.

Ethanol Extract: The results of the mutagenesis assay for the ethanol extract are presented in Tables 5 through 8. The first trial of the assay using the ethanol extract failed due to high background (extraction blank) mutant frequencies. The data from that assay were recorded but not reported. The assay was repeated using a freshly prepared extract. In the non-activated system, cultures treated with a test article extract concentration of 10 $\mu\text{L}/\text{mL}$ were cloned and produced suspension growths of 102% and 105%. In the S9-activated system cultures treated with a test article extract concentration of 10 $\mu\text{L}/\text{mL}$ were cloned and produced suspension growths of 105% and 106%. No test article precipitate was observed in treatment medium.

No treated cultures exhibited mutant frequencies that were at least 55 mutants per 10^6 clonable cells over that of the extraction blank control. The total growths were 98% and 93% for the non-activated cultures and 100% and 90% for the S9-activated cultures at a test article extract concentration of 10 μ L/mL.

CONCLUSION

All criteria for a valid study were met as described in the protocol. The results of the L5178Y/TK⁺ Mouse Lymphoma Mutagenesis Assay indicate that, under the conditions of this study, saline and ethanol extracts of 300cc Siltex Moderate Profile Gel-filled Mammary Prosthesis were concluded to be negative.

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REFERENCES

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- Kozak, C.A. and Ruddle, F.H. (1977) Assignment of the genes for thymidine kinase and galactokinase to *Mus musculus* chromosome 11 and the preferential segregation of this chromosome in Chinese hamster/mouse somatic cell hybrids. *Somatic Cell Genet.* 3:121-133.
- Moore, M.M., Clive, D., Howard, B.E., Batson, A.G. and Turner, N.T. (1985) In situ analysis of trifluorothymidine-resistant (TFT^r) mutants of L5178Y/TK^{+/+} mouse lymphoma cells. *Mutation Research* 151:147-159.
- OECD Guideline 476 (Genetic Toxicology: In Vitro Mammalian Cell Gene Mutation Tests), Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, published by OECD, Paris, February 1998.
- USP (XXIV), (88) Biological Reactivity Test, *In Vivo*, p. 1832-1836.

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TABLE 1

CLONING DATA FOR L5178Y/TK⁺ MOUSE LYMPHOMA CELLS
TREATED WITH 300cc Siltex Moderate Profile Gel-filled Mammary Prosthesis
IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION
Saline Extract

| Test Article Concentration (µL/mL) | TFT Colonies | | | | VC Colonies | | | | Mutant Freq. ^a | Induced Mutant Freq. ^b | % Total Growth ^c |
|--|--------------|------|--------|---------|-------------|------|-----|---------|---------------------------|-----------------------------------|-----------------------------|
| | Counts | Mean | Counts | Mean | Counts | Mean | | | | | |
| Solvent 1 | 34 | 81 | 38 | 51 ±21 | 172 | 139 | 183 | 165 ±19 | 62 | | |
| Solvent 2 | 24 | 93 | 123 | 80 ±41 | 188 | 188 | 171 | 182 ±8 | 88 | | |
| Mean Solvent Mutant Frequency= 75 | | | | | | | | | | | |
| 100 A | 67 | 68 | 53 | 63 ±7 | 195 | 196 | 209 | 200 ±6 | 63 | -12 | 107 |
| 100 B | 93 | 39 | 44 | 59 ±24 | 240 | 218 | 192 | 217 ±20 | 54 | -21 | 111 |
| ----- | | | | | | | | | | | |
| Positive Control - Methyl Methanesulfonate (µg/mL) | | | | | | | | | | | |
| 10 | 214 | 195 | 137 | 182 ±33 | 151 | 135 | 144 | 143 ±7 | 254 | 179 | 50 |
| 20 | 219 | 225 | 210 | 218 ±6 | 73 | 58 | 70 | 67 ±6 | 651 | 576 | 16 |

Solvent = saline extraction blank

A and B or 1 and 2 are duplicate cultures

^a - Mutant frequency (per 10⁶ surviving cells) = $\frac{\text{Average \# TFT colonies}}{\text{average \# VC colonies}} \times 200$

^b - Induced mutant frequency per 10⁶ surviving cells) = $\frac{\text{mutant frequency} - \text{average mutant frequency of solvent controls}}$

^c - % total growth = $\frac{(\% \text{ suspension growth} \times \% \text{ cloning growth})}{100}$

TABLE 2

**TOTAL COMPOUND TOXICITY DATA FOR L5178Y/TK⁺ MOUSE LYMPHOMA CELLS
TREATED WITH 300cc Siltex Moderata Profile Gel-filled Mammary Prosthesis
IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION
Saline Extract**

| Test Article Concentration ($\mu\text{L}/\text{mL}$) | | Cell Concentration ($\times 10^6$) | | Susp Growth | | Cloning Growth | | % Total Growth ^d |
|--|---|---|-------|--------------------|--------------------|----------------|--------------------|--------------------------------|
| | | Day 1 | Day 2 | Total ^a | %Cntl ^b | Avg VC | %Cntl ^c | |
| Solvent | 1 | 1.384 | 1.701 | 26.2 | | 165 | | |
| Solvent | 2 | 1.409 | 1.566 | 24.5 | | 182 | | |
| 100 | A | 1.397 | 1.517 | 23.5 | 93 | 200 | 115 | 107 |
| 100 | B | 1.389 | 1.463 | 22.6 | 89 | 217 | 125 | 111 |

Positive Control - Methyl Methanesulfonate ($\mu\text{g}/\text{mL}$)

| | | | | | | | |
|----|-------|-------|------|----|-----|----|----|
| 10 | 1.093 | 1.255 | 15.2 | 60 | 143 | 83 | 50 |
| 20 | 0.973 | 0.948 | 10.2 | 40 | 67 | 39 | 16 |

Solvent = saline extraction blank

A and B or 1 and 2 are duplicate cultures

$$^a - \text{Total suspension growth} = \frac{\text{Day 1 cell conc.}}{0.3 \times 10^6 \text{ cells/mL}} \times \frac{\text{Day 2 cell conc.}}{\text{Day 1 adjusted cell conc.}}$$

$$^b - \% \text{ of control suspension growth} = \frac{\text{total treatment suspension growth}}{\text{average solvent control total suspension growth}} \times 100$$

$$^c - \% \text{ control cloning growth} = \frac{\text{average VC of treated culture}}{\text{average VC of solvent control}} \times 100$$

$$^d - \% \text{ total growth} = \frac{(\% \text{ suspension growth})(\% \text{ cloning growth})}{100}$$

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TABLE 3

**CLONING DATA FOR L5178Y/TK⁺ MOUSE LYMPHOMA CELLS
TREATED WITH 300cc Siltex Moderate Profile Gel-filled Mammary Prosthesis
IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION
Saline Extract**

| Test Article Concentration (µL/mL) | TFT Colonies | | | | VC Colonies | | | | Mutant Freq. ^a | Induced Mutant Freq. ^b | % Total Growth ^c |
|---|--------------|------|--------|---------|-------------|------|--------|---------|------------------------------|---|-----------------------------------|
| | Counts | Mean | Counts | Mean | Counts | Mean | Counts | Mean | | | |
| Solvent 1 | 30 | 67 | 59 | 52 ±16 | 191 | 155 | 185 | 177 ±16 | 59 | | |
| Solvent 2 | 77 | 37 | 24 | 46 ±23 | 192 | 182 | 192 | 189 ±5 | 49 | | |
| Mean Solvent Mutant Frequency= 54 | | | | | | | | | | | |
| 100 A | 101 | 81 | 94 | 92 ±8 | 184 | 168 | 194 | 182 ±11 | 101 | 47 | 106 |
| 100 B | 42 | 40 | 42 | 41 ±1 | 205 | 198 | 203 | 202 ±3 | 41 | -13 | 116 |
| ----- | | | | | | | | | | | |
| Positive Control - 7,12 Dimethylbenz(a)anthracene (µg/mL) | | | | | | | | | | | |
| 2.5 | 131 | 163 | 172 | 155 ±18 | 135 | 105 | 116 | 119 ±12 | 262 | 208 | 54 |
| 4 | 291 | 260 | 139 | 230 ±66 | 101 | 119 | 117 | 112 ±8 | 409 | 356 | 40 |

Solvent = saline extraction blank

A and B or 1 and 2 are duplicate cultures

^a - Mutant frequency (per 10⁶ surviving cells) = $\frac{\text{Average \# TFT colonies}}{\text{average \# VC colonies}} \times 200$

^b - Induced mutant frequency (per 10⁶ surviving cells) = $\frac{\text{mutant average mutant frequency}}{\text{frequency of solvent controls}}$

^c - % total growth = $\frac{(\% \text{ suspension growth} \times \% \text{ cloning growth})}{100}$

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TABLE 4

**TOTAL COMPOUND TOXICITY DATA FOR L5178Y/TK⁺ MOUSE LYMPHOMA CELLS
TREATED WITH 300cc Siltex Moderate Profile Gel-filled Mammary Prosthesis
IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION
Saline Extract**

| Test Article Concentration (µL/mL) | Cell Concentration (X 10 ⁶) | Cell Concentration | | Susp Growth | | Cloning Growth | | % Total Growth ^d |
|--|--|--------------------|-------|--------------------|--------------------|----------------|--------------------|--------------------------------|
| | | Day 1 | Day 2 | Total ^a | %Cntl ^b | Avg VC | %Cntl ^c | |
| Solvent 1 | 1 | 0.945 | 1.360 | 14.3 | | 177 | | |
| Solvent 2 | 2 | 0.901 | 1.514 | 15.2 | | 189 | | |
| 100 A | | 0.921 | 1.539 | 15.8 | 107 | 182 | 100 | 106 |
| 100 B | | 0.944 | 1.480 | 15.5 | 105 | 202 | 110 | 116 |

Positive Control - 7,12 Dimethylbenz(a)anthracene (µg/mL)

| | | | | | | | |
|-----|-------|-------|------|----|-----|----|----|
| 2.5 | 0.726 | 1.512 | 12.2 | 83 | 119 | 65 | 54 |
| 4 | 0.646 | 1.332 | 9.6 | 65 | 112 | 61 | 40 |

Solvent = saline extraction blank

A and B or 1 and 2 are duplicate cultures

$$^a - \text{Total suspension growth} = \frac{\text{Day 1 cell conc.}}{0.3 \times 10^6 \text{ cells/mL}} \times \frac{\text{Day 2 cell conc.}}{\text{Day 1 adjusted cell conc.}}$$

$$^b - \% \text{ of control suspension growth} = \frac{\text{total treatment suspension growth}}{\text{average solvent control total suspension growth}} \times 100$$

$$^c - \% \text{ control cloning growth} = \frac{\text{average VC of treated culture}}{\text{average VC of solvent control}} \times 100$$

$$^d - \% \text{ total growth} = \frac{(\% \text{ suspension growth})(\% \text{ cloning growth})}{100}$$

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TABLE 5

**CLONING DATA FOR L5178Y/TK⁺ MOUSE LYMPHOMA CELLS
TREATED WITH 300cc Siltex Moderate Profile Gel-filled Mammary Prosthesis
IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION
Ethanol Extract**

| Test Article Concentration (μ L/mL) | TFT Colonies | | | | VC Colonies | | | | Mutant Freq. ^a | Induced Mutant Freq. ^b | % Total Growth ^c |
|--|--------------|------|--------|--------------|-------------|------|--------|--------------|------------------------------|---|-----------------------------------|
| | Counts | Mean | Counts | Mean | Counts | Mean | Counts | Mean | | | |
| Solvent 1 | 38 | 46 | 19 | 34 \pm 11 | 96 | 116 | 120 | 111 \pm 10 | 62 | | |
| Solvent 2 | 20 | 20 | 54 | 31 \pm 16 | 212 | 149 | 81 | 147 \pm 53 | 43 | | |
| Mean Solvent Mutant Frequency= 52 | | | | | | | | | | | |
| 10 A | 47 | 31 | 18 | 32 \pm 12 | 99 | 148 | 124 | 124 \pm 20 | 52 | -1 | 98 |
| 10 B | 15 | 20 | 25 | 20 \pm 4 | 80 | 109 | 156 | 115 \pm 31 | 35 | -18 | 93 |
| ----- | | | | | | | | | | | |
| Positive Control - Methyl Methanesulfonate (μ g/mL) | | | | | | | | | | | |
| 10 | 218 | 84 | 124 | 142 \pm 56 | 91 | 92 | 55 | 79 \pm 17 | 358 | 306 | 49 |
| 20 | 120 | 89 | 79 | 96 \pm 17 | 28 | 27 | 52 | 36 \pm 12 | 538 | 486 | 14 |

Solvent = ethanol extraction blank

A and B or 1 and 2 are duplicate cultures

^a - Mutant frequency (per 10⁶ surviving cells) = $\frac{\text{Average \# TFT colonies}}{\text{average \# VC colonies}} \times 200$

^b - Induced mutant frequency (per 10⁶ surviving cells) = $\frac{\text{mutant frequency} - \text{average mutant frequency of solvent controls}}$

^c - % total growth = $\frac{(\% \text{ suspension growth} \times \% \text{ cloning growth})}{100}$

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TABLE 6

TOTAL COMPOUND TOXICITY DATA FOR L5178Y/TK⁺ MOUSE LYMPHOMA CELLS
TREATED WITH 300cc Siltex Moderate Profile Gel-filled Mammary Prosthesis
IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION
Ethanol Extract

| Test Article Concentration (µL/mL) | Cell Concentration (X 10 ⁶) | Cell Concentration | | Susp Growth | | Cloning Growth | | % Total Growth ^d |
|--|---|--------------------|-------|--------------------|--------------------|----------------|--------------------|-----------------------------|
| | | Day 1 | Day 2 | Total ^a | %Cntl ^b | Avg VC | %Cntl ^c | |
| Solvent 1 | 1.135 | 1.446 | 18.2 | | 111 | | | |
| Solvent 2 | 1.161 | 1.496 | 19.3 | | 147 | | | |
| 10 A | 1.158 | 1.485 | 19.1 | 102 | 124 | 96 | 98 | |
| 10 B | 1.186 | 1.494 | 19.7 | 105 | 115 | 89 | 93 | |
| ----- | | | | | | | | |
| Positive Control - Methyl Methanesulfonate (µg/mL) | | | | | | | | |
| 10 | 0.973 | 1.379 | 14.9 | 79 | 79 | 61 | 49 | |
| 20 | 0.821 | 1.037 | 9.5 | 50 | 36 | 28 | 14 | |

Solvent = ethanol extraction blank

A and B or 1 and 2 are duplicate cultures

$$^a - \text{Total suspension growth} = \frac{\text{Day 1 cell conc.}}{0.3 \times 10^6 \text{ cells/mL}} \times \frac{\text{Day 2 cell conc.}}{\text{Day 1 adjusted cell conc.}}$$

$$^b - \% \text{ of control suspension growth} = \frac{\text{total treatment suspension growth}}{\text{average solvent control total suspension growth}} \times 100$$

$$^c - \% \text{ control cloning growth} = \frac{\text{average VC of treated culture}}{\text{average VC of solvent control}} \times 100$$

$$^d - \% \text{ total growth} = \frac{(\% \text{ suspension growth})(\% \text{ cloning growth})}{100}$$

000192

TABLE 7

**CLONING DATA FOR L5178Y/TK⁺ MOUSE LYMPHOMA CELLS
TREATED WITH 300cc Siltex Moderate Profile Gel-filled Mammary Prosthesis
IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION
Ethanol Extract**

| Test Article Concentration (μ L/mL) | TFT Colonies | | | | VC Colonies | | | | Mutant Freq. ^a | Induced Mutant Freq. ^b | % Total Growth ^c |
|---|--------------|------|--------|--------------|-------------|------|--------|--------------|------------------------------|---|-----------------------------------|
| | Counts | Mean | Counts | Mean | Counts | Mean | Counts | Mean | | | |
| Solvent 1 | 45 | 35 | 18 | 33 \pm 11 | 132 | 109 | 130 | 124 \pm 10 | 53 | | |
| Solvent 2 | 27 | 21 | 31 | 26 \pm 4 | 116 | 120 | 123 | 120 \pm 3 | 44 | | |
| Mean Solvent Mutant Frequency= 48 | | | | | | | | | | | |
| 10 A | 37 | 41 | 34 | 37 \pm 3 | 133 | 135 | 78 | 115 \pm 26 | 65 | 16 | 100 |
| 10 B | 25 | 48 | 30 | 34 \pm 10 | 86 | 105 | 117 | 103 \pm 13 | 67 | 18 | 90 |
| ----- | | | | | | | | | | | |
| Positive Control - 7,12 Dimethylbenz(a)anthracene (μ g/mL) | | | | | | | | | | | |
| 2.5 | 79 | 186 | 118 | 128 \pm 44 | 86 | 80 | 104 | 90 \pm 10 | 284 | 235 | 55 |
| 4 | 240 | 132 | 139 | 170 \pm 49 | 112 | 112 | 80 | 101 \pm 15 | 336 | 288 | 47 |

Solvent = ethanol extraction blank

A and B or 1 and 2 are duplicate cultures

^a - Mutant frequency (per 10⁶ surviving cells) = $\frac{\text{Average \# TFT colonies}}{\text{average \# VC colonies}} \times 200$

^b - Induced mutant frequency (per 10⁶ surviving cells) = $\frac{\text{mutant frequency}}{\text{average mutant frequency of solvent controls}}$

^c - % total growth = $\frac{(\% \text{ suspension growth} \times \% \text{ cloning growth})}{100}$

000193

TABLE 8

**TOTAL COMPOUND TOXICITY DATA FOR L5178Y/TK⁺ MOUSE LYMPHOMA CELLS
TREATED WITH 300cc Siltex Moderate Profile Gel-filled Mammary Prosthesis
IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION
Ethanol Extract**

| Test Article Concentration ($\mu\text{L}/\text{mL}$) | Cell Concentration ($\times 10^6$) | Cell Concentration | | Susp Growth | | Cloning Growth | | % Total Growth ^d |
|---|---|--------------------|-------|--------------------|--------------------|----------------|--------------------|--------------------------------|
| | | Day 1 | Day 2 | Total ^a | %Cntl ^b | Avg VC | %Cntl ^c | |
| Solvent 1 | 0.723 | 1.515 | 12.2 | | 124 | | | |
| Solvent 2 | 0.827 | 1.490 | 13.7 | | 120 | | | |
| 10 A | 0.796 | 1.543 | 13.6 | 105 | 115 | 95 | 100 | |
| 10 B | 0.801 | 1.545 | 13.7 | 106 | 103 | 84 | 90 | |
| ----- | | | | | | | | |
| Positive Control - 7,12 Dimethylbenz(a)anthracene ($\mu\text{g}/\text{mL}$) | | | | | | | | |
| 2.5 | 0.602 | 1.438 | 9.6 | 74 | 90 | 74 | 55 | |
| 4 | 0.528 | 1.255 | 7.4 | 57 | 101 | 83 | 47 | |

Solvent = ethanol extraction blank

A and B or 1 and 2 are duplicate cultures

$$^a - \text{Total suspension growth} = \frac{\text{Day 1 cell conc.}}{0.3 \times 10^6 \text{ cells/mL}} \times \frac{\text{Day 2 cell conc.}}{\text{Day 1 adjusted cell conc.}}$$

$$^b - \% \text{ of control suspension growth} = \frac{\text{total treatment suspension growth}}{\text{average solvent control total suspension growth}} \times 100$$

$$^c - \% \text{ control cloning growth} = \frac{\text{average VC of treated culture}}{\text{average VC of solvent control}} \times 100$$

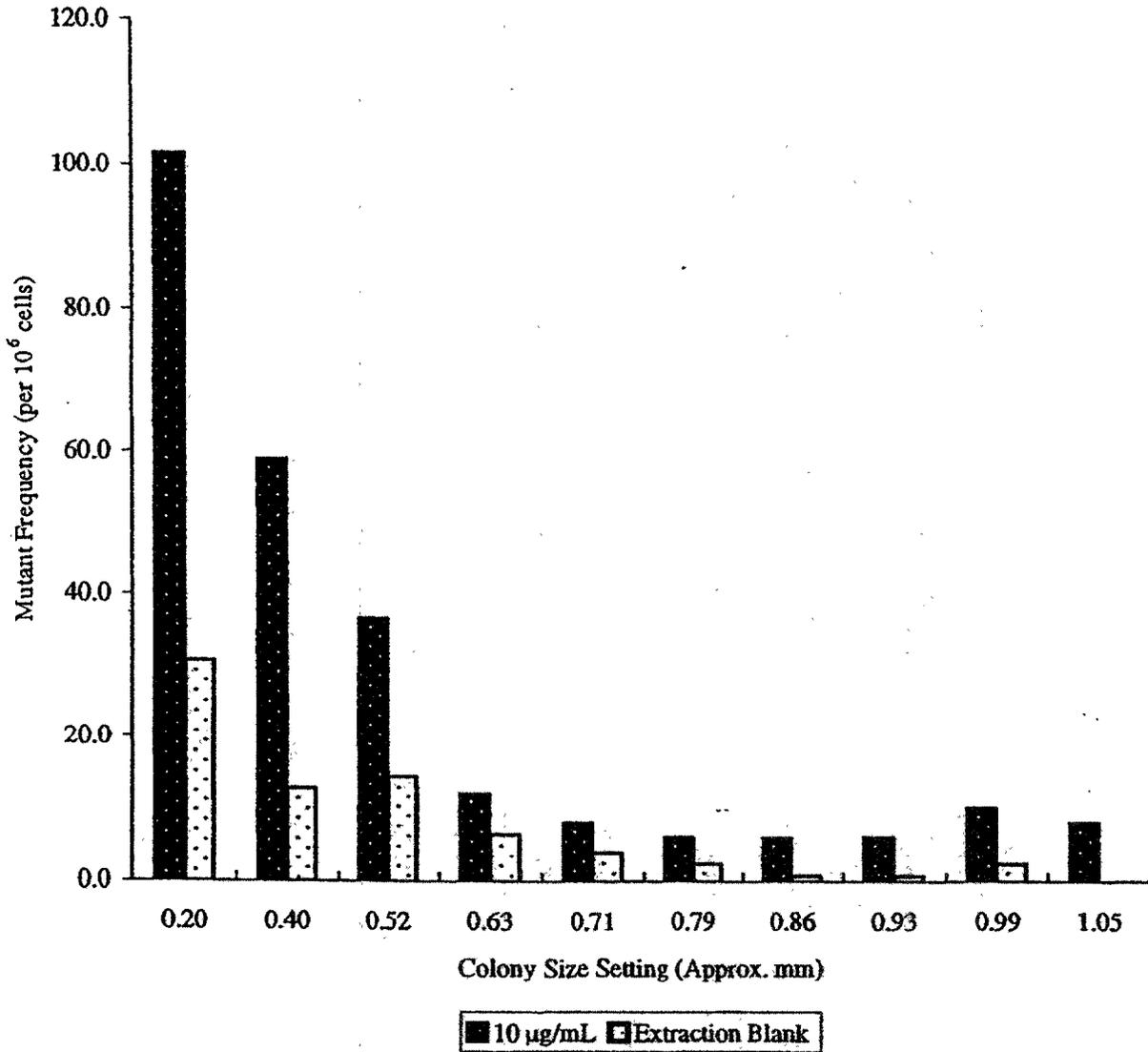
$$^d - \% \text{ total growth} = \frac{(\% \text{ suspension growth})(\% \text{ cloning growth})}{100}$$

000194

Figure 1

Colony Size Distribution in the Absence of Metabolic Activation
(Positive Control Compared with Saline Extraction Blank Control)

AA73XP.702201 B1 MMS

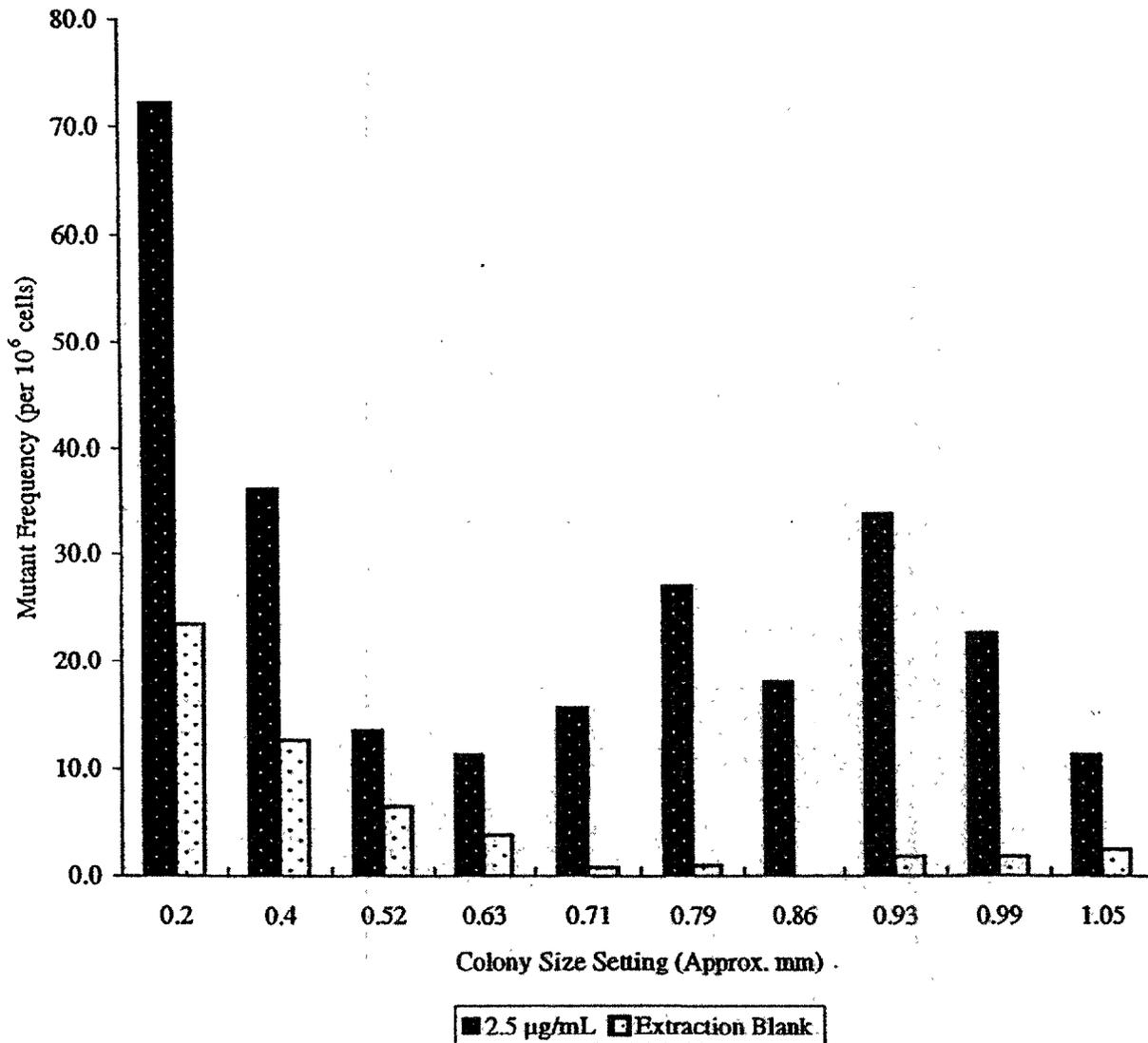


000195

Figure 2

Colony Size Distribution in the Presence of Metabolic Activation
(Positive Control Compared with Saline Extraction Blank Control)

AA73XP.702201 B1 DMBA

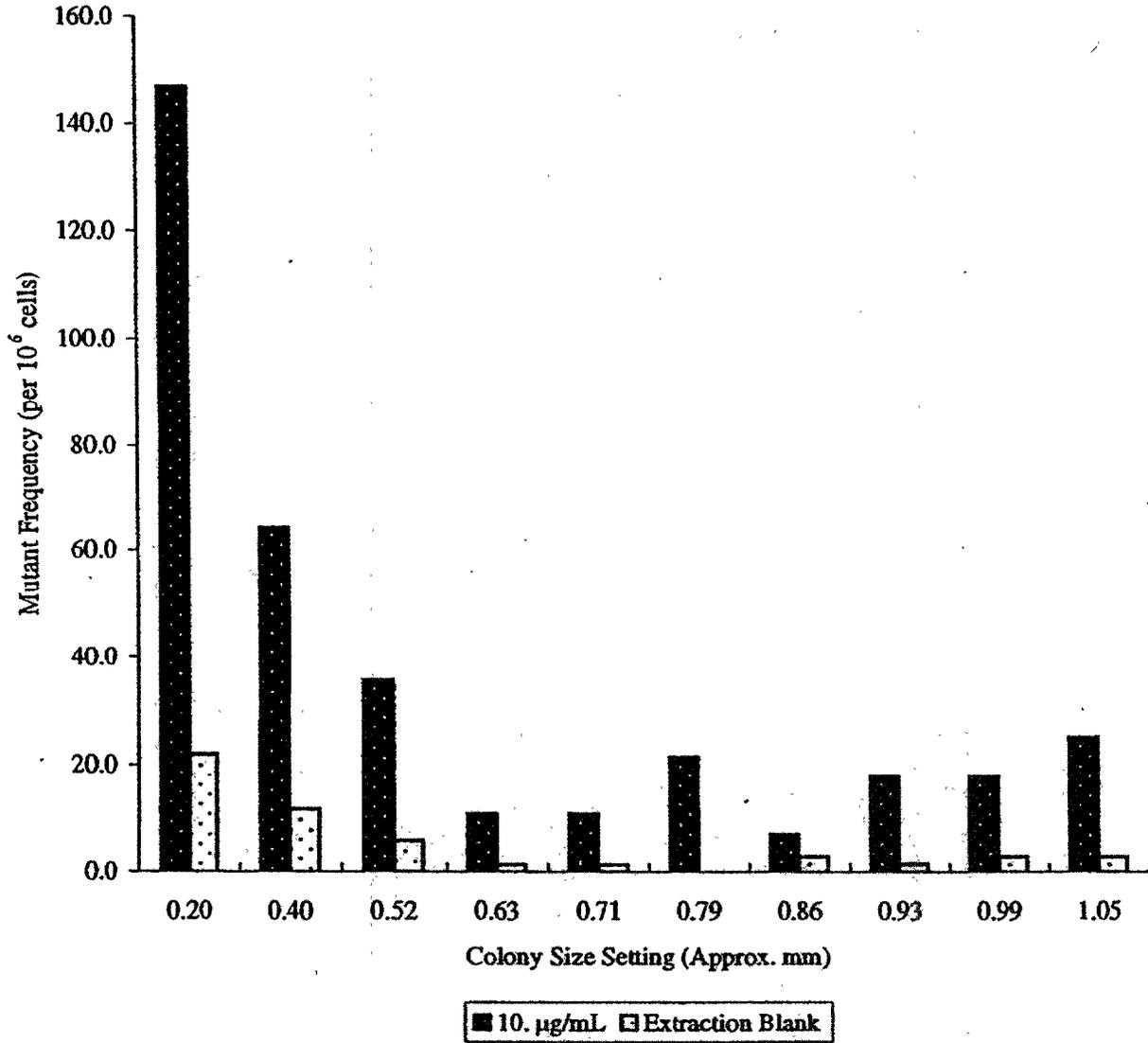


0000796

Figure 3

Colony Size Distribution in the Absence of Metabolic Activation
(Positive Control Compared with Ethanol Extraction Blank Control)

AA73XP.702201.BTL B2 MMS

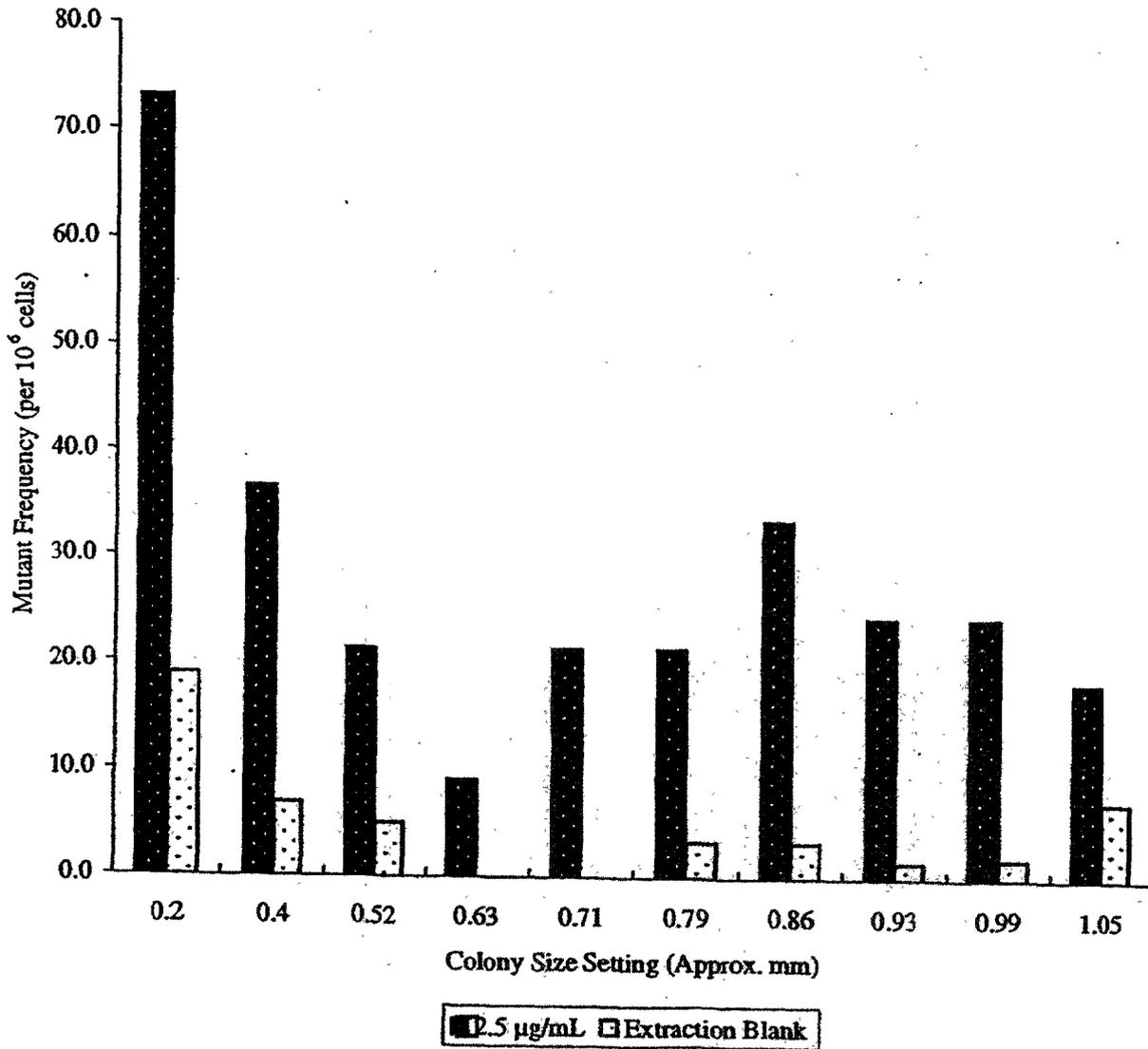


000197

Figure 4

Colony Size Distribution in the Presence of Metabolic Activation
(Positive Control Compared with Ethanol Extraction Blank Control)

AA73XP.702201.BTL B2 DMBA



000198

APPENDIX I:
Historical Control Data

000199

Mouse Lymphoma Historical Control Data

2000 - 2002

| | Non-activated (4-Hour) | | | Non-Activated (24-Hour) | | |
|---------|------------------------|--------------|--------------|-------------------------|---------------|---------------|
| | Solvent Control | 10 µg/mL MMS | 20 µg/mL MMS | Solvent Control | 2.5 µg/mL MMS | 5.0 µg/mL MMS |
| Mean MF | 51.9 | 311.6 | 626.6 | 46.5 | 197.8 | 340.1 |
| SD | 19.1 | 114.6 | 212.7 | 17.9 | 67.2 | 106.3 |
| Maximum | 100 | 940 | 1214 | 95 | 397 | 641 |
| Minimum | 20 | 62 | 122 | 20 | 79 | 113 |

| | S9-Activated (4-Hour) | | | | |
|---------|-----------------------|----------------|----------------|----------------|----------------|
| | Solvent Control | 1.0 µg/mL DMBA | 1.5 µg/mL DMBA | 2.5 µg/mL DMBA | 4.0 µg/mL DMBA |
| Mean MF | 58.1 | 365.4 | 453.2 | 294.4 | 362.3 |
| SD | 20.5 | 217.4 | 388.6 | 223.0 | 112.4 |
| Maximum | 100 | 1027 | 2051 | 1715 | 748 |
| Minimum | 23 | 132 | 107 | 63 | 161 |

Solvent control (Fischer's medium, distilled water, saline, DMSO, ethanol, acetone or vehicle supplied by Sponsor)

- MMS Methyl methanesulfonate
- DMBA Dimethylbenz(a)anthracene
- MF Mutant frequency per 10⁶ clonable cells
- SD Standard deviation

000200

**APPENDIX II:
Study Protocol**

000201

Received by RA/OA 4/3/03

QA 8054/4/03
APPROVED

BioReliance Study Number: AA131P.702201.BTL

**In Vitro Mammalian Cell Gene Mutation Test
Conducted with Test Article Extracts**

1.0 PURPOSE

The purpose of this study is to evaluate the mutagenic potential of saline and ethanol extracts of the test article based on quantitation of forward mutations at the thymidine kinase locus of L5178Y mouse lymphoma cells.

2.0 SPONSOR

- 2.1 Name: **Mentor Corporation**
- 2.2 Address: 201 Mentor Drive
Santa Barbara, CA 93111
- 2.3 Representative: Philip Yang
Phone: 805-879-6427
FAX: 805-879-6014
Email: pyang@mentorcorp.com
- 2.4 Sponsor Project #: (NONE)

3.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

- 3.1 Test Article: **300cc Siltex Moderate Profile Gel-filled Mammary Prosthesis**
(Cat. #354-3007, Lot #257949) made with [REDACTED] materials, [REDACTED] patches, new calender, [REDACTED] mandrels, and [REDACTED] sterilization. The sterile device was cut open to extract both the gel and the shell.
- 3.2 Controls: Negative: Saline extraction blank
Ethanol extraction blank
Positive: Methyl methanesulfonate (MMS)
7,12-dimethylbenz(a)anthracene (DMBA)
- 3.3 Determination of Strength, Purity, etc.

Unless alternate arrangements are made, the testing facility at BioReliance will not perform analysis of the dosing solutions. The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test article, and the stability and strength of the test article in the solvent (or vehicle)

3.4 Test Article Retention Sample

The retention of a reserve sample of the test article will be the responsibility of the Sponsor.

4.0 TESTING FACILITY AND KEY PERSONNEL

- 4.1 Name: Toxicology Testing Facility
BioReliance
- 4.2 Address: 9630 Medical Center Drive
Rockville, MD 20850
- 4.3 Study Director: Richard H. C. San, Ph.D.
Phone: (301) 610-2222
Fax: (301) 738-2362
E-mail: rsan@bioreliance.com

5.0 TEST SCHEDULE

- 5.1 Proposed Experimental Initiation Date: 15 Apr 2003
- 5.2 Proposed Experimental Completion Date: 29 Apr 2003
- 5.3 Proposed Report Date: 26 May 2003

6.0 TEST SYSTEM

L5178Y/TK^{+/+} mouse lymphoma cells are heterozygous at the normally diploid thymidine kinase (TK) locus. L5178Y/TK^{+/+}, clone 3.7.2C, were received from Dr. Patricia Poorman-Allen, Glaxo Wellcome Inc., Research Triangle Park, North Carolina. Each freeze lot of cells has been tested and found to be free of mycoplasma contamination. This system has been demonstrated to be sensitive to the mutagenic activity of a variety of chemicals.

7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

The mammalian mutation assay will be performed by exposing duplicate cultures of L5178Y/TK^{+/+} cells to one concentration of each test article extract as well as positive and negative (extraction blank) controls. Exposures will be for 4 hours in the presence and absence of an S9 activation system. Following a two-day expression period, with daily cell population adjustments, cultures will be cloned, in triplicate, in restrictive medium containing soft agar to select for the mutant phenotype. After a 10 to 14 day selection period, mutant colonies will be enumerated. The mutagenic potential of the test article will be measured by its ability to induce TK^{+/+} → TK^{-/-} mutations. For those test articles demonstrating a positive response, mutant colonies will be sized as an indication of mechanism of action.

7.1 Extract Preparation

The Sponsor has specified that extracts of the test article will be prepared using saline and ethanol according to the following procedures (USP (XXIV), <88> Biological Reactivity Test, *In Vivo*, p. 1832-1836). The test article will be extracted with 20 mL extraction medium for each of the following specimen sizes: 120 cm² total surface area (both sides) for specimens 0.5 mm or less in thickness or 60 cm² total surface area (both sides) for specimens >0.5 mm in thickness. When surface area cannot be readily determined due to the configuration of the specimen, an extraction ratio based on 0.1 g of elastomer or 0.2 g of plastic or other polymers for every 1 mL of extracting fluid will be used. The test article will be placed in the extraction vessel and immersed in the required volume of extraction medium. The test article may be cut into small pieces to facilitate total immersion in the extraction medium. The headspace of the extraction vessel will be purged with nitrogen gas and the vessel capped tightly. The extraction mixture will be shaken for 72±2 hours at 50±2°C. At the end of the extraction period, the vessel will be cooled to room temperature and shaken vigorously for several minutes. The extract will be aseptically decanted into a dry, sterile vessel, and stored at room temperature. Saline and ethanol extraction blanks will be prepared in the same manner, without the addition of test article. All extracts and extraction blanks will be used within 24 hours of preparation.

Unless specified otherwise, dilutions of the test article extracts will be prepared by dilution with the extraction blank immediately prior to use. All test article dosing will be at room temperature under yellow light.

7.2 Dose Levels

The test article extracts will be administered full strength (undiluted) into the culture medium. The dose level will be limited by the maximum concentration of extraction medium compatible with the test system, i.e., 10% final concentration for saline and 1% final concentration for ethanol.

7.3 Route and Frequency of Administration

Cell cultures will be treated for 4 hours, both in the presence and absence of metabolic activation.

7.4 Exogenous Metabolic Activation

Aroclor 1254-induced rat liver S9 will be used as the metabolic activation system. The source of S9 will be adult male Sprague-Dawley rats induced by a single injection of Aroclor 1254 at a dose level of 500 mg/kg body weight five days prior to sacrifice. The S9 will be batch prepared and stored frozen at approximately -70°C until used.

Immediately prior to use, the S9 will be thawed and mixed with a cofactor pool to contain 11.25 mg DL-isocitric acid, 6 mg NADP, and 0.25 mL S9 homogenate per ml in Fischer's Media for Leukemic Cells of Mice with 0.1% Pluronics (F₀P). The S9 mix will be adjusted to pH 7. The S9 mix will be adjusted to pH 7. Each 10 mL culture will contain 4 mL S9 mix (final S9 concentration of 10%).

7.5 Controls

7.5.1 Negative Control

The extraction blank will be used as the negative control. The final concentration of the extraction medium in treatment medium will not exceed 10% for saline and 1% for ethanol.

7.5.2 Positive Controls

Results obtained from treatment with these articles will be used to assure responsiveness of the test system but not to provide a standard for comparison with the test article.

Methyl methanesulfonate (MMS) will be used at two concentrations between 1.0 and 20 µg/mL as the positive control for the non-activated test system and to determine that the assay is capable of detecting small colonies. For the S9-activated system, 7,12-dimethylbenz(a)anthracene (DMBA) will be used at two or three concentrations between 0.5 and 10 µg/mL.

7.6 Preparation of Target Cells

Prior to use in the assay, L5178Y/TK^{+/+} cells will be cleansed to reduce the frequency of spontaneously occurring TK⁺ cells. Using the procedure described by Clive and Spector (1975), L5178Y cells will be cultured for 24 hours in the presence of thymidine, hypoxanthine, methotrexate and glycine to poison the TK⁺ cells.

L5178Y/TK^{+/+} cells will be prepared in 50% conditioned F₁₀P and 50% F₀P.

7.7 Identification of the Test System

Using a permanent marking pen, the treatment tubes will be identified by the study number and a code system to designate the treatment condition and test phase.

7.8 Treatment of Target Cells

Treatment will be carried out in conical tubes by combining 6×10^6 L5178Y/TK^{+/+} cells and 1 mL dosing solution of saline test article extract or saline extraction blank (or 100 µL of ethanol test article extract, ethanol extraction blank or positive control) in a total volume of 10 mL F₀P medium or S-9 activation mixture. Treatment tubes will be gassed with $5 \pm 1\%$ CO₂ in air, capped tightly, and

incubated with mechanical mixing for 4 hours at 37±1°C. The preparation and addition of the test article dosing solutions will be carried out under amber lighting and the cells will be incubated in the dark during the 4-hour exposure period.

7.9 Expression of the Mutant Phenotype

At the end of the exposure period, the cells will be washed twice with F₀P or F₁₀P and collected by centrifugation. The cells will be resuspended in 20 mL F₁₀P, gassed with 5±1% CO₂ in air and cultured in suspension at 37±1°C for two days following treatment. Cell population adjustments to 0.3 x 10⁶ cells/mL will be made at 24 and 48 hours post treatment.

7.10 Selection of the Mutant Phenotype

For selection of the trifluorothymidine (TFT)-resistant phenotype, cells from up to ten treatment conditions demonstrating from 0% to 90% suspension growth inhibition will be plated into three replicate dishes at a density of 1 x 10⁶ cells/100mm plate in cloning medium containing 0.23% agar and 2-4 µg TFT/mL. For estimation of cloning efficiency at the time of selection, 200 cells/100mm plate will be plated in triplicate in cloning medium free of TFT (viable cell (VC) plate). Plates will be incubated at 37±1°C in a humidified atmosphere of 5±1% CO₂ for 10-14 days.

The total number of colonies per plate will be determined for the VC plates and the total relative growth calculated. The total number of colonies per TFT plate will then be determined for those cultures with ≥10% total growth. Colonies are enumerated using an automatic counter; if the automatic counter cannot be used, the colonies will be counted manually. The diameters of the TFT colonies from the positive control and solvent control cultures will be determined over a range of approximately 0.2 to 1.1 mm. In the event the test article extract demonstrates a positive response, the diameters of the TFT colonies for the test article extract will be determined over a range of approximately 0.2 to 1.1 mm.

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

8.1 Negative Controls

The spontaneous mutant frequency of the extraction blank cultures must be within 20 to 100 TFT-resistant mutants per 10⁶ surviving cells. The cloning efficiency of the extraction blank group also must be greater than 50%.

8.2 Positive Controls

At least one concentration of each positive control must exhibit mutant frequencies of ≥100 mutants per 10⁶ clonable cells over the background level. The colony size distribution for the MMS positive control must show an increase in both small and large colonies (Moore *et al.*, 1985; Aaron *et al.*, 1994).

9.0 EVALUATION OF TEST RESULTS

The cytotoxic effects of each treatment condition are expressed relative to the solvent-treated control for suspension growth over two days post-treatment and for total growth (suspension growth corrected for plating efficiency at the time of selection). The mutant frequency for each treatment condition is calculated by dividing the mean number of colonies on the TFT-plates by the mean number of colonies on the VC-plates and multiplying by the dilution factor (2×10^4), and is expressed as TFT-resistant mutants per 10^6 surviving cells.

In evaluation of the data, increases in mutant frequencies which occur only at highly toxic concentrations (i.e., less than 10% total growth) are not considered biologically relevant. All conclusions will be based on sound scientific judgement; however, the following criteria are presented as a guide to interpretation of the data (Clive *et al.*, 1995):

- A result will be considered positive if a dose level with 10% or greater total growth exhibits a mutant frequency of ≥ 100 mutants per 10^6 clonable cells over the background level.
- A result will be considered equivocal if the mutant frequency in treated cultures is between 55 and 99 mutants per 10^6 clonable cells over the background level.
- A result will be considered negative if the mutant frequency in treated cultures is fewer than 55 mutants per 10^6 clonable cells over the background level.

10.0 REPORT

A report of the results of this study will be prepared by the Testing Laboratory and will accurately describe all methods used in the generation and analysis of data.

The report will include, but not be limited to:

- Test substance: identification and CAS no., if known; physical nature and purity, if known; physicochemical properties relevant to the conduct of the study, if known; stability of test article, if known.
- Extraction Media: justification for choice of media; details on test article extraction
- Cell type used, number of cultures, methods for maintenance of cell cultures
- Rationale for selection of concentrations and number of cultures
- Test conditions: composition of media, CO_2 concentration, concentration of test article extract, vehicle, incubation temperature, incubation time, duration of treatment, cell density during treatment, type of metabolic activation system, positive and negative controls, length of expression period, selective agent

- Method used to enumerate numbers of viable and mutant colonies and the number of colonies in each plate
- Dose-response relationship, if applicable
- Distribution of the mutant colony diameter for the solvent and positive controls and, when the test article induces a positive response, for at least one dose level of the test article (the highest positive concentration)
- Positive and solvent control historical data
- Discussion
- Conclusion

11.0 RECORDS AND ARCHIVES

All raw data, protocol, and a copy of all reports will be maintained according to Standard Operating Procedure OPQP3040 by the BioReliance RAQA unit headquartered at BioReliance, 14920 Broschart Road, Rockville, MD 20850. Per this SOP, paper records will be retained for at least three years after which time the Sponsor will be contacted for a decision as to the final disposition of the materials. All study materials returned to the Sponsor or destroyed will first be copied and the copy will be retained in the BioReliance archives for a minimum of 10 years. Raw data, the protocol and reports generated at facilities other than BioReliance will be archived per the contractual arrangements between that facility and the Sponsor.

12.0 REGULATORY REQUIREMENTS/GOOD LABORATORY PRACTICE

This protocol has been written to comply with OECD Guideline 476 (Genetic Toxicology: *In Vitro* Mammalian Cell Gene Mutation Tests), February 1998; and the EPA Health Effects Testing Guidelines, Subpart 870.5300 (Detection of Gene Mutations in Somatic Cells in Culture), August 1998.

The portions of this study conducted at BioReliance will be performed in compliance with the provisions of the Good Laboratory Practice Regulations for Nonclinical Laboratory Studies (GLPs). The protocol, an in-process phase, the raw data, and report(s) will be audited per the Standard Operating Procedures (SOPs) of BioReliance by the Quality Assurance Unit of BioReliance for compliance with GLPs, the SOPs of BioReliance and the study protocol. The in-process inspection will be performed to audit the critical assay procedures and systems supporting the assay. A signed QA statement will be included in the final report. This statement will list the system phases inspected during the previous quarter or the study-specific phases, the dates of each inspection, and the dates the results of each inspection were reported to the Study Director and the Study Director's management. In addition, a signed GLP compliance statement will be included in the final report. This statement will cite the GLP guideline(s) with which the study is

compliant and any exceptions to this compliance, if applicable, including the omission of characterization or stability analyses of the test or control articles or their mixtures.

Raw data, the protocol and reports generated at facilities other than BioReliance will or will not be QA audited per the contractual arrangements between that facility and the Sponsor.

Will this study be submitted to a regulatory agency? Yes

If so to which agency or agencies? USFDA, Japan, Australia, EU

Unless arrangements are made to the contrary, unused dosing solutions will be disposed of following administration to the test system and all residual test article will be disposed of following finalization of the report.

13.0 REFERENCES

Aaron, C.S., Bolcsfoldi, G., Glatt, H.-R., Moore, M., Nishi, Y., Stankowski, L., Theiss, J. and Thompson, E. (1994). Mammalian cell gene mutation assays working group report. *Mutation Research* 312:235-239.

Clive, D., Bolcsfoldi, G., Clements, J., Cole, J., Homna, M., Majeska, J., Moore, M., Muller, L., Myhr, B., Oberly, T., Oudelhkim, M., Rudd, C., Shimada, H., Sofuni, T., Thybaud, V. and Wilcox, P. (1995). Consensus agreement regarding protocol issues discussed during the mouse lymphoma workshop: Portland, Oregon, May 7, 1994. *Environ. Molec. Mutagen.* 25:165-168.

Clive, D. and Spector, J.F.S. (1975). Laboratory procedure for assessing specific locus mutations at the TK locus in cultured L5178Y mouse lymphoma cells. *Mutation Research* 31:17-29.

EPA Health Effects Testing Guidelines, Subpart 870.5300 (Detection of Gene Mutations in Somatic Cells in Culture), August 1998.

Moore, M.M., Clive, D., Howard, B.E., Batson, A.G. and Turner, N.T. In situ analysis of trifluorothymidine-resistant (TFT) mutants of L5178Y/TK^{+/+} mouse lymphoma cells. (1985). *Mutation Research* 151:147-159.

OECD Guideline 476 (Genetic Toxicology: *In Vitro* Mammalian Cell Gene Mutation Tests), Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, published by OECD, Paris, February 1998.

USP (XXIV), (88) Biological Reactivity Test, *In Vivo*, p. 1832-1836.

14.0 APPROVAL

Philip S. Yang
Sponsor Representative

3/26/03
Date

PHILIP S. YANG
(Print or Type Name)

Richard X
BioReliance Study Director

03 Apr 2003
Date

Dal. Jacobson
BioReliance Study Management

03 Apr 2003
Date